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TOWNSEND and TOWNSEND and CREW LLP

By: /Terrie J. Rau/

PATENT
Attorney Docket No. 080306-000100US
Client Ref. No. P16809

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:

Ralph Mocikat

Application No.: 10/716,580

Filed: November 18, 2003

For: EXPRESSION OF IMMUNOGLOBULIN-CYTOKINE FUSION PROTEINS IN MALIGNANT B CELLS

Customer No.: 20350

Confirmation No. 6256

Examiner: WOODWARD, Cherie Michelle

Technology Center/Art Unit: 1647

APPELLANT'S BRIEF UNDER 37 CFR §41.37

Mail Stop Appeal Brief
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Commissioner:

This brief is filed pursuant to 37 C.F.R. §41.37, following the Notice of Appeal received by the USPTO on June 4, 2009. Also submitted with this brief is a petition to extend time to response for one month (from August 4, 2009, to September 4, 2009) and authorization to pay the fee as set forth in 37 C.F.R. §41.20(b)(2).

TABLE OF CONTENTS

I. REAL PARTY IN INTEREST

The real party in interest in U.S. Application No. 10/716,580 is GSF-Forschungszentrum fur Unwelt und Gesundheit, Oberschleissheim, Germany.

II. RELATED APPEALS AND INTERFERENCES

There are no other pending appeals by Appellant or interferences in which Appellant is involved, the outcome of which would directly affect the decision by the Board of Patent Appeals and Interferences in this pending appeal.

III. STATUS OF CLAIMS

Claims 1-30 were originally filed. Subsequently, claims 6, 10, 18-28, and 30 were canceled. Claims 1-5, 7-9, 11-17, and 29 are currently under examination. In the Final Office Action mailed January 6, 2009, claims 1-5, 7-9, and 12-17 are rejected under 35 U.S.C. §112, first paragraph, for alleged inadequate written description. Claims 1-5, 7-9, 11, 13-17, and 29 are rejected for alleged anticipation under 35 U.S.C. §102(e) by the Polack reference as evidenced by the Mucke reference. Claims 1-5, 7-9, 11-13, and 15-17 are rejected under 35 U.S.C. §103 for alleged obviousness over Polack in view of the Levy reference and the Gillies reference. Claims 1-5, 7-9, and 11-17 are further rejected under 35 U.S.C. §103 for alleged obviousness over the Polack or Mucke reference in view of the Mocikat reference. The rejections of claims 1-5, 7-9, 11-17, and 19 are being appealed.

IV. STATUS OF AMENDMENTS

Claim 1 was amended subsequent to the Final Office Action of January 6, 2009. The word "continuous" is added in line 3 of claim 1, line 1 of claim 2, and line 1 of claim 3. The amendment is entered as indicated on page 2 of the Advisory Action mailed June 3, 2009. Support for the recited term of "a continuous region of at least 1.5 kb" can be found throughout the entire specification. For example, in the last paragraph on page 9, it is stated that the vector of this invention has "a region of at least 1.5 kb with homology to an intron region"; in the last

paragraph on page 10, it is stated that, "[t]he homologous sequence contained in said vector must have a length of at least 1.5 kb to achieve a homologous recombination event at all." These statements make it clear that it is one single region, *i.e.*, a continuous, undisrupted region, of a length of at least 1.5 kb that is necessary to ensure the recombination event. Furthermore, the concept of a continuous region of at least 1.5 kb is also presented in German patent document DE 44 06 512, which is incorporated by reference in this application (see the second full paragraph on page 9). The present application indicates that the starting material for the construction of the vectors of this invention was the integration vectors described in DE 44 06 512. See page 8, the first full paragraph, of the present application. DE 44 06 512 in turn describes in Example 1 that the overall homology flank had a length of 3.0 kb and then a 1.0 kb fragment was excised. In other words, the remaining homologous region was a 2.0 kb continuous sequence. As such, this application provides inherent support for the claim language "a continuous region of at least 1.5 kb."

V. SUMMARY OF CLAIMED SUBJECT MATTER

The claimed subject matter in this appeal relates to a vector for expressing immunoglobulin-cytokine fusion proteins in malignant B cells. The vector comprises the following components operably linked to each other: (a) a continuous region of at least 1.5 kb that is homologous to an at least 1.5 kb segment of the μ intron or the *k* intron; (b) at least one DNA sequence encoding a constant region of an immunoglobulin or a part of the constant region; (c) a DNA sequence encoding a cytokine; and (d) a marker gene that is selectable in eukaryotic B cells and contains a functional enhancer region. Also claimed is a malignant B cell containing the above-described vector in integrated form, wherein an immunoglobulin-cytokine fusion protein is expressed by said cell.

Therefore, the present invention describes a recombinant ***integration*** vector useful for inducing a tumor-specific immune response against B-cell lymphoma, by way of a fusion protein of a cytokine and a tumor-specific idiotype. Rather than a traditional expression vector directly encoding the fusion protein, which would require individual cloning of the idiotype of every patient's lymphoma cells, the integration vector of this invention includes a continuous

sequence of at least 1.5 kb that is homologous to an at least 1.5 kb segment of the μ intron or the k intron, such that, following transfection of a B lymphoma cell and subsequent homologous recombination, the DNA sequences coding a cytokine and a immunoglobulin constant region (or a part thereof) also present in the integration vector are incorporated into the malignant B cell's genomic sequence. A cytokine fusion protein is then produced by the B cell to include the specific idiotype encoded by the endogenous sequence of the malignant B cell. After rendered incapable of proliferation, such a malignant B cell expressing a tumor immunoglobulin-cytokine fusion protein can be reintroduced into a patient to elicit a specific anti-B cell lymphoma immunity due to enhanced recruitment of antigen-presenting cells by the cytokine and more effective presentation of the tumor-specific immunoglobulin idiotype. This invention eliminates the need to clone each patient's idiotypic domain and is thus quick, convenient, and less expensive.

Claim 1

The subject matter claimed in independent claim 1 is a vector for the expression of immunoglobulin-cytokine fusion proteins in malignant B cells. The vector comprises these components operably linked to each other: (a) a continuous region of at least 1.5 kb that is homologous to an at least 1.5 kb segment of the μ intron or the k intron; (b) at least one DNA sequence encoding a constant region of an immunoglobulin or a part of the constant region; (c) a DNA sequence encoding a cytokine; and (d) a marker gene which is selectable in eukaryotic B cells and contains a functional enhancer region. Support for this claim can be found in the specification as filed, *e.g.*, in original claim 1 on page 1, lines 8-17; in original claim 6 on page 2, lines 7-8; page 10, lines 8-9; and in the last paragraph on page 9.

VI. GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

A. The rejection of claims 1-5, 7-9, and 11-17 under 35 U.S.C. §112, first paragraph, for alleged inadequate written description.

B. The rejection of claims 1-5, 7-9, 11, 13-17, and 29 under 35 U.S.C. §102(e) for alleged anticipation by Polack as evidenced by Mucke.

C. The rejection of claims 1-5, 7-9, 11-13, and 15-17 under 35 U.S.C. §103 for alleged obviousness over Polack, Levy, and Gillies as evidenced by Mucke.

D. The rejection of claims 1-5, 7-9, and 11-17 under 35 U.S.C. §103 for alleged obviousness over Mucke, Polack, and Mocikat.

VII. ARGUMENTS

A. The Written Description Rejection Is Improper

Claims 1-5, 7-9, and 11-17 are rejected under 35 U.S.C. §112, first paragraph, for alleged inadequate written description. Appellant respectfully traverses the rejection and requests its reversal.

According to the MPEP, to satisfy the written description requirement, a patent specification must describe the claimed invention in sufficient detail such that one skilled in the art can reasonably conclude that the inventor had possession of the claimed invention at the time of filing. Possession of a claimed invention may be demonstrated by description of the invention with all of its limitations using such descriptive means as words, structures, figures, diagrams, and formulas that fully set forth the claimed invention. MPEP §2163 I.

The pending claims are drawn to a recombination vector for expressing immunoglobulin-cytokine fusion proteins in malignant B cells. The vector comprises the following components operably linked to each other: (a) a continuous region of at least 1.5 kb that is homologous to an at least 1.5 kb segment of ***the μ intron*** or ***the k intron***; (b) at least one DNA sequence encoding ***a constant region of an immunoglobulin or a part of the constant region***; (c) a DNA sequence encoding ***a cytokine***; and (d) ***a marker gene*** that is selectable in eukaryotic B cells and contains a functional ***enhancer*** region.

Appellant takes the position that, at the effective filing date of this application, all of these common components of the claimed vector--*μ* or *k* intron sequences, immunoglobulin constant region sequences, cytokine sequences, selectable marker sequences, and enhancer sequences, were well known and available to a person of ordinary skill in the art. An artisan

upon reading the present disclosure would therefore reasonably conclude that the inventor had in his possession these components and therefore the claimed vector. As such, the present disclosure meets the written description requirement under 35 U.S.C. §112, first paragraph, which requires a patent specification to describe the claimed invention in sufficient detail such that one skilled in the art can reasonably conclude that the inventor had possession of the claimed invention at the time of filing.

On page 2 of the Final Office Action mailed January 6, 2009, the Examiner cites *University of Rochester v. G.D. Searles & Co.*, 69 USPQ2d 1886 (Fed. Cir. 2004) and *Ex parte Kubin*, 83 USPQ2d 1410 (Bd. Pat. App. & Int. 2007) and argues that the written description requirement is not met merely by demonstrating one's ability to possess the claimed invention. The Examiner stresses the distinction between the ability to obtain the invention and the inventor's being in possession of the invention. Appellant understands this distinction and, based on this understanding, Appellant contends that the instant disclosure, especially in light of the state of the art, clearly shows both a skilled artisan's ability to obtain the invention and inventor's being in possession of the invention.

It must be pointed out that the fact pattern in this application is very different from that of *Rochester* or *Kubin*. In *Rochester*, the claims in question were directed to the use of a COX-2 inhibitor. Although the patent applicant provided a detailed, enabling description for a skilled artisan to identify such COX-2 inhibitors, they were neither known in the art nor named in the application. The court held that the written description requirement was not met. In *Kubin*, the application claimed a genus of nucleotide sequences encoding natural killer cell activation inducing ligand ("NAIL") polypeptides, which were purportedly new and were defined as having at least 80% identity to a reference amino acid sequence and binding to CD48. The specification provided two species of nucleic acids and three species of fusion polypeptides within the claim scope, but none of the exemplary NAIL sequences included any variation within the reference amino acid sequence. While acknowledging the teaching in the specification on how to make and test additional NAIL sequences within the claim scope, the Board ruled that the exemplary species did not sufficiently represent the claimed genus and that the application fails

to provide adequate written description to show applicant had possession of the full scope of the claimed invention at the time of filing.

In direct contrast to *Rochester* or *Kubin*, the genera of μ or *k* intron sequences, immunoglobulin constant region sequences, cytokine sequences, selectable marker sequences, or enhancer sequences were well known in the art. The Examiner's analysis of "representative number of species" or "common structural features" and conclusion pertaining to a patent applicant's attempt to broadly claim a genus of previously unknown species thus have no relevance in the written description assessment in this application.

In short, the written description rejection stands for the proposition that an artisan of ordinary skill would not be convinced of Appellant's possession of the claimed vector, even though the components of the ***vectors***, ***μ or k intron sequences***, ***immunoglobulin constant region sequences***, ***cytokine sequences***, ***selectable marker sequences***, and ***enhancer sequences***, were all known in the art at the time this application was filed. This is not a tenable position.

In the Final Office Action mailed January 6, 2009, the Examiner argues that the immunoglobulin μ or κ sequences are long and contain various segments such as V, J, and C. See the last paragraph on page 5. Regardless of their length, these are known sequences (which the Examiner does not seem to dispute); the large number of possible selections within each genus of ***known*** components is evidence of a broad scope of enablement and possession of the invention, not remotely akin to the "hunting" for the unknown referred to by the Supreme Court in *Brenner v. Manson*, 148 USPQ 689 (S. Ct. 1966). Contrary to the Examiner's position, one's ability to choose from a broad range of ***known*** components to practice a claimed invention in fact supports the conclusion of inventor's having possession of the invention.

Accordingly, it is respectfully submitted that the instant application has fully met the written description requirement under 35 U.S.C. §112, first paragraph. Appellant therefore respectfully requests that the written description rejection be reversed.

B. The Anticipation Rejection Is Improper

Claims 1-5, 7-9, 11, 13-17, and 29 are rejected under 35 U.S.C. §102(e) for alleged anticipation by the Polack reference as evidenced by the Mucke reference. Appellant respectfully requests the reversal of this rejection.

To anticipate a pending claim, a prior art reference must provide, either expressly or implicitly, each and every limitation of the pending claim. MPEP §2131. The pending claims are drawn to a vector for the expression of immunoglobulin-cytokine fusion proteins in malignant B cells. Among other features, the vector comprises "a continuous region of at least 1.5 kb that is homologous to an at least 1.5 kb segment of the μ intron or the *k* intron."

The Polack reference and Mucke reference describe expression vectors that contain a promoter as well as a polynucleotide sequence encoding the polypeptide to be expressed from the vectors. Appellant contends that neither Polack nor Mucke provides all limitations of the pending claims. For instance, the limitation of "a continuous region of at least 1.5 kb which is homologous to an at least 1.5 kb segment of the μ intron or the *k* intron" cannot be found in either of the two references. As the Examiner has acknowledged, Polack describes the combined use of two enhancer κ intron elements, which provide a combined length of over 1.5 kb but each is less than 1.5 kb, see, *e.g.*, paragraph 4 on page 7 of the Office Action mailed April 3, 2008. Because neither Polack nor Mucke provides the limitation of "a continuous region of at least 1.5 kb which is homologous to an at least 1.5 kb segment of the μ intron or the *k* intron," there is no basis for the anticipation rejection.

As such, the rejection under 35 U.S.C. §102(e) is improper and must be reversed.

C. The Obviousness Rejection over Polack, Levy, and Gillies Is Improper

Claims 1-5, 7-9, 11-13, and 15-17 are rejected under 35 U.S.C. §103 for alleged obviousness over Polack (evidenced by Mucke) in view of the Levy reference and the Gillies reference. Appellant respectfully requests reversal of the rejection.

In order to establish a *prima facie* showing of obviousness, three requirements must be satisfied: all limitations of a pending claim must be expressly or impliedly disclosed by

prior art references; there must be a suggestion or motivation in the art for one skilled artisan to combine the limitations; and there must be a reasonable expectation of success in making such a combination. MPEP §2143.

As discussed in the last section, the primary references by Polack *et al.* and Mucke *et al.* relate to expression vectors and fail to provide at least one limitation of the pending claims, namely "a continuous region of at least 1.5 kb that is homologous to an at least 1.5 kb segment of the μ intron or the *k* intron." The secondary references, Levy and Gillies, are cited to provide teaching of a vector encoding an idiotype/GM-CSF fusion protein and a vector encoding a recombinant antibody-cytokine fusion protein, respectively. Since the Examiner has not identified anything in the secondary references, Levy and Gillies, that would supplement the missing claim limitation of "a continuous region of at least 1.5 kb that is homologous to an at least 1.5 kb segment of the μ intron or the *k* intron," no *prima facie* obviousness is established.

Reversal of the obviousness rejection over Polack in view of Levy and Gillies is therefore requested.

D. The Obviousness Rejection over Polack/Mucke and Mocikat Is Improper

Claims 1-5, 7-9, and 11-17 are further rejected under 35 U.S.C. §103 for alleged obviousness over Polack or Mucke in view of the Mocikat reference. Appellant respectfully traverses the rejection and requests its reversal.

As discussed in the last section, the primary references by Polack *et al.* and Mucke *et al.* relate to expression vectors and fail to provide at least one limitation of the pending claims, namely "a continuous region of at least 1.5 kb that is homologous to an at least 1.5 kb segment of the μ intron or the *k* intron." The secondary reference by Mocikat is cited to provide teaching of a vector for homologous recombination at the Ig locus. The Examiner argues that, since Mocikat uses a 2.3 kb fragment of the mouse μ intron sequence in their recombination vector, the missing claim limitation is supplemented. The Examiner further argues that Mocikat provides a motivation to combine its teaching with that of Polack, because Mocikat allegedly teaches various advantageous features of this 2.3 kb mouse μ intron fragment (see the last

paragraph on page 10 and also the paragraph bridging pages 11 and 12 of the Final Office Action).

Appellant disagrees with the Examiner's arguments for at least two reasons: first, because of fundamental differences in the purpose and mechanism of action between expression vectors (*e.g.*, the Polack vector) and integration vectors (*e.g.*, the Mocikat vector)[1], there would be no motivation for a skilled artisan to combine the teaching of the Polack and Mocikat references. The advantages in protein expression that the Examiner has alleged Mocikat's 2.3 kb mouse μ intron sequence would confer relate solely to the expression of rearranged genomic sequence resulted from homologous recombination and genomic integration, crucial for an integration vector to effectuate gene expression as intended. The recombination/integration process, however, is completely irrelevant to the direct expression of a recombinant protein, which is how an expression vector such as the Polack vector works. Therefore, the advantages of the 2.3 kb mouse μ intron fragment present in Mocikat's integration vector will not provide any motivation for a skilled artisan to use the fragment in Polack's expression vector.

Second, if anything is suggested by Polack, Mucke, and Mocikat together, it is to teach away from any consideration of replacing the enhancers used by Polack with the 2.3 kb μ intron sequence used by Mocikat to modify Polack's expression vector. This is because the Polack vector is an expression vector and uses enhancers to promote the expression of a coding sequence carried by the vector. Replacing the enhancers with the 2.3 kb intron sequence would completely defeat the purpose of enhancing/promoting expression. As such, there is at least one strong incentive for a skilled artisan to NOT combine Polack, Mucke, and Mocikat. With the

[1] The vector of the present invention is fundamentally different from Polack's vector: the vector of this invention is an integration vector, which carries a cytokine coding sequence but does not directly express the cytokine; instead, the μ or k intron homologous sequence within the vector directs a recombination event, causing the cytokine coding sequence provided in the integration vector to be integrated into a host tumor cell's genome and joined with a genomic sequence encoding a tumor-specific idiotype. The fusion protein of cytokine/tumor-specific idiotype is then expressed from the "new" genomic sequence resulted from the recombination. In contrast, Polack's vector is a conventional expression vector containing a promoter and a polynucleotide sequence encoding a polypeptide, so that the polypeptide is directly expressed from the vector. Polack's vector does not cause any targeted genomic recombination/integration, and does not become a part of the host genomic sequence.

cited references "teaching away" from the claimed invention, Appellant contends that no *prima facie* obviousness has been or can be established.

Accordingly, reversal of the obviousness rejection based on Polack/Mucke and Mocikat is respectfully requested.

VIII. CONCLUSION

In view of the above discussion, Appellant respectfully submits that the written description rejection, anticipation rejection, and obviousness rejections of the pending claims are erroneous and therefore requests their reversal.

Respectfully submitted,

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62168910 v1

IX. CLAIMS APPENDIX

1. A vector for the expression of immunoglobulin-cytokine fusion proteins in malignant B cells, comprising the following components operably linked to each other

(a) a continuous region of at least 1.5 kb which is homologous to an at least 1.5 kb segment of the μ intron or the k intron;

(b) at least one DNA sequence encoding a constant region of an immunoglobulin or a part of the constant region;

(c) a DNA sequence encoding a cytokine; and

(d) a marker gene which is selectable in eukaryotic B cells and contains a functional enhancer region.

2. The vector according to claim 1, wherein said continuous region of at least 1.5 kb contains a functional C_μ or C_k enhancer.

3. The vector according to claim 1, wherein said continuous region of at least 1.5 kb contains a non-functional C_μ or C_k enhancer.

4. The vector according to claim 1, wherein the marker gene selectable in eukaryotic B cells contains a non-functional enhancer.

5. The vector according to claim 1, wherein the marker gene selectable in eukaryotic B cells lacks an enhancer.

7. The vector according to claim 1, wherein the region homologous to a region comprising the C_μ or the C_k enhancer of the μ or the k intron comprises at least 1.9 kb.

8. The vector according to claim 1, wherein the region homologous to a region comprising the C_μ or the C_k enhancer of the u or the k intron comprises at least 2.0 kb.

9. The vector according to claim 1, said vector containing a regulatory unit which is compatible with bacteria.

11. The vector according to claim 1, wherein the DNA sequence of (b) encodes the constant region of a human immunoglobulin.

12. The vector according to claim 1, wherein the DNA sequence of (b) encodes the constant region of a mouse, rat, goat, horse or sheep immunoglobulin.

13. The vector according to claim 1, wherein the DNA sequence of (b) encodes the constant region of a secretory antibody.

14. The vector according to claim 1, wherein the DNA sequence according to (b) encodes the constant region of a membrane-bound antibody.

15. The vector according to claim 1, characterized in that said DNA sequence of (c) encodes an interleukin, an interferon, a colony-stimulating factor, a lymphokine, or a growth factor.

16. The vector according to claim 15, characterized in that said DNA sequence of (c) encodes IL-2, IL-4, IL-7, IL-12, IL-13, GM-CSF or interferon γ.

17. The vector according to claim 1, wherein the selectable marker gene is gpt, neo, or a marker gene encoding hygromycin resistance.

29. A malignant B cell containing a vector according to claim 1 in integrated form, wherein an immunoglobulin-cytokine fusion protein is expressed by said cell.

X. EVIDENCE APPENDIX

This appendix contains a copy of each of the Polack, Mucke, Levy, Gillies, and Mocikat references.

XI. RELATED PROCEEDINGS APPENDIX

None.

US006521449B1

(12) **United States Patent**
Polack et al.

(10) **Patent No.: US 6,521,449 B1**
(45) **Date of Patent: Feb. 18, 2003**

(54) **GENE CONSTRUCT AND ITS USE**

(75) Inventors: **Axel Polack**, München (DE); **Konstanze Hörtnagel**, München (DE); **Jürgen Wolf**, Köln (DE); **Susanne Mücke**, Köln (DE)

(73) Assignee: **GSF-Forschungszentrum für Umwelt und Gesundheit GmbH** (DE)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

(21) Appl. No.: **09/434,574**

(22) Filed: **Nov. 4, 1999**

Related U.S. Application Data

(63) Continuation of application No. 08/713,089, filed on Sep. 12, 1996, now abandoned.

(30) **Foreign Application Priority Data**

Nov. 7, 1995 (DE) 195 41 450

(51) **Int. Cl.**[7] **C12N 15/63**; C12N 15/85; C12N 15/86; C07H 21/04; A61K 48/00; A61K 9/127

(52) **U.S. Cl.** **435/320.1**; 435/325; 536/24.1; 530/350; 424/450; 514/44

(58) **Field of Search** 435/320.1, 458, 435/440, 325; 530/350; 536/23.5, 23.4; 424/450; 514/2, 44

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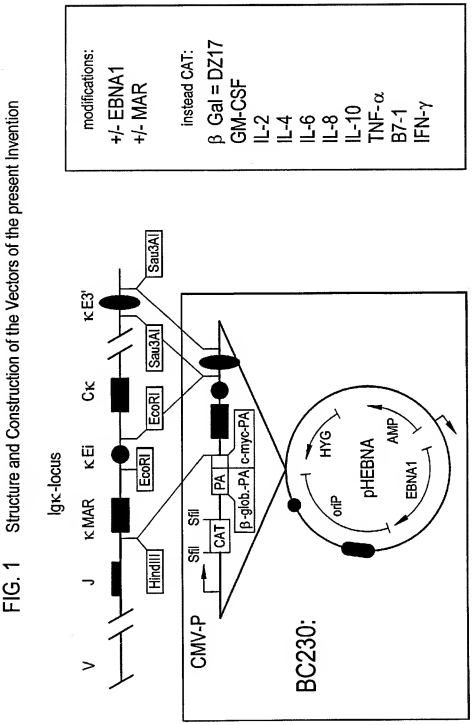
Primary Examiner—Michael C. Wilson
(74) *Attorney, Agent, or Firm*—Bingham McCutchen LLP; David W. Maher

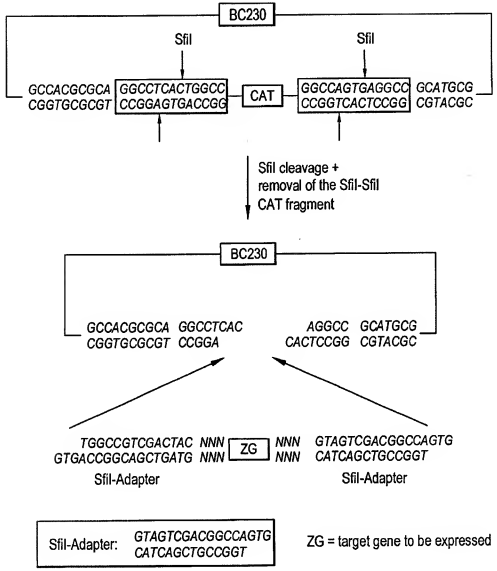
(57) **ABSTRACT**

Compositions of matter and methods for expressing a polypeptide are provided. In one aspect, a gene construct is provided that comprises an enhancer comprising particular elements or combinations of elements from the immunoglobulin κ locus, the immunoglobulin heavy chain μ locus, and the immunoglobulin λ locus, and a promoter and polyadenylation site linked to a nucleotide sequence encoding a polypeptide from a select group. Cells comprising the gene construct, processes for producing such cells, and processes for producing the polypeptide encoded by the gene construct from such cells are also provided.

36 Claims, 3 Drawing Sheets

FIG. 1 Structure and Construction of the Vectors of the present Invention





schematic representation of cloning of a gene to be expressed in vector BC230 or a derivative thereof

FIG.2

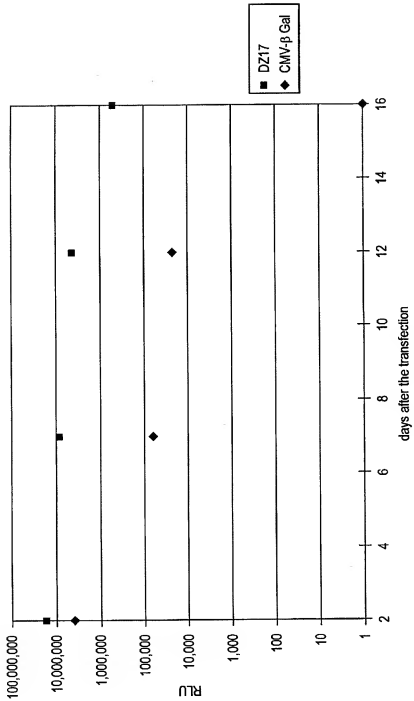

β-galactosidase activity in BL cells transfected with DZ17 and CMV-β GAL, respectively
RLU
100,000,000
10,000,000
1,000,000
100,000
10,000
1,000
100
10
1
2
4
6
8
10
12
14
16
days after the transfection
DZ17
CMV-β Gal

FIG.3

GENE CONSTRUCT AND ITS USE

This application is a continuation of U.S. application Ser. No. 08/713,059, filed Sep. 12, 1996 (now abandoned). Priority is claimed under 35 U.S.C. 119 to German patent application number 19541450.0 filed Nov. 7, 1995.

The present invention relates to a gene construct, a pharmaceutical preparation and their use.

The beginning of the era of gene therapy in medicine has been marked by the successful gene transfer of the adenosine desaminase gene to a child with severe immunodeficiency. To date findings coming mainly from animal experiments indicate that this form of therapy is not only useful in the correction of genetically caused diseases but also in the therapy of malignant neoplasias (Culver and Blaese, 1994).

The methods up to now being in the test phase are aimed at either a direct destruction or at least "normalization" of the tumor cell, or at the activation of an immune reaction directed against the tumor. The destruction by the transfer of so-called suicide genes or the normalization by the transfer of tumor suppressor genes requires the gene transfer being performed with high efficiency. The direct intratumour transfer of murine cell lines producing retroviruses containing the suicide gene thymidine kinase of herpes virus has been already performed in the case of multiform glioblastoma (Culver and Van Gilder, 1994).

As long as there is no efficient system available to achieve targeted gene transfer to all tumor cells in vivo, approaches involving the immune system in tracking down and destroying all tumor cells seem the most promising. However, a prerequisite of these approaches is the fundamental capability of the immune system to recognize the tumor cells by means of tumor-specific antigens, which appear on tumor cells and not on normal cells (Boon et al., 1994). For example, these tumor-specific antigens include viral gene products (e. g. gene products of the human papilloma virus in genital tumors) or mutationally altered oncogene products (e. g. the ras gene product or the tumor-specific bcr-abl fusion protein). Further suitable candidates for tumor specific antigens are the so-called idiotypes, i. e. immunoglobulins or T cell receptors on the cellular surfaces of B or T cell derived tumors. Recently, the identification of a plurality of tumor-associated antigens has been carried out, for example in malignant melanoma. These genes, however, are not exclusively expressed by the tumor, but to a small extent also by other somatic cells, such as melanocytes. The knowledge of tumor-specific or tumor-associated antigens, respectively, is probably about to increase sharply because of the recent successful recovery and analysis by biochemical methods of the peptides presented by a tumor MHC complex (Mandelboim et al.; Cox et al., 1994).

Already in the mid-eighties, using an approach employing experiments on laboratory animals tumor cells were observed to loose their tumorigenicity in the syngeneic animal if the tumor cells were transfected with a cytokine expression vector (e. g. IL-2) by. gene transfer (Pardoll, 1993). This effect has also been observed in the case of a mixture of modified (i.e., cells that contained the expression vector) and non-modified cells. The local production of an immunostimulatory cytokine in a subset of the tumor cells is obviously capable of causing an immune reaction directed against the wild-type tumor. One of the most extensive studies of this kind using the murine malignant melanoma model B-16 showed retroviruses transducing GM-CSF, IL-4, and IL-6 to be most effective (Dranoff et al., 1993). The outcome of these observations was that a number of clinical studies on the subject of intratumour transfer of

cytokines have been entered or are presently being entered worldwide (Foa et al., 1994). Generally, the protocols entail an ex vivo gene transfer into tumor cells which have been established in vitro for a short time period. In most of the protocols amphotrophic retroviruses are employed as vectors. After viral gene transfer, the cells are reimplanted into the patients. They can be irradiated prior to reimplantation. These approaches are, however, strongly limited by the technical difficulties of culturing the tumor cells in vitro even for a short time period. Therefore, a modification of this approach entails transducing or transfecting, respectively, either tumor infiltrating lymphocytes (TIL) (Treisman et al., 1995) or autologous fibroblast cells instead of the tumor cells themselves.

A further approach also referring to observations obtained from animal experiments in the eighties has been developed by Gary Nabel and already converted into a clinical protocol (Nabel et al., 1993; Plautz et al., 1993). This approach assumes that artificial allogenization of a subset of the tumor cells by the transfer of transplantation antigens may be sufficient to induce an immune reaction of the organism against the unmodified tumor cell. This protocol entails the direct transfer of an HLA B7 expression construct into the tumor using liposomes. Repeated injection of the HLA B7 gene construct into skin metastases of a moribund patient brought about regression of another untreated metastasis and of a pulmonary mestastasis, respectively (Nabel et al., 1993).

Many in vivo tumor cells lack the B7 surface antigen mediating co-stimulatory signals for T cell recognition. Therefore, attempts are made to stimulate the production of this signalling molecule in tumor cells by gene transfer (June et al., 1994; Li et al., 1994).

The aforementioned approaches to solve the problems bear the following disadvantages:

Retroviral Vectors

An advantage of amphotropic retroviruses is that integration of the proviral DNA into the target cell and the viral promoter/-enhancer combination generally permit a stable expression level during several cell divisions. The essential step of integration, however, bears the danger of insertional mutagenesis. Moreover, so-called "packaging" cell lines produce relatively small amounts of recombinant virus which to date fail to be enriched because of their lability. Therefore, direct intratumour gene transfer is only possible using virus producing cells. Release of infectious virus in the target organism, however, may lead to infection of other dividing cells, such as intestinal epithelium or hematopoietic stem cells, after hematogenous transmission.

Direct Intratumour DNA Transfer

The liposome-mediated direct incorporation of DNA has been demonstrated successfully using the endothelium of large blood vessels (Ohno et al., 1994). An advantage of this approach is that it lacks the risk of insertional mutagenesis as well as of the undesirable remote effect; but this approach achieves only transient expression of the incorporated gene construct in dividing tissue because the DNA generally fails to be integrated or replicated.

Cell Culture of Tumor Cells and Ex VIVO Transfection

One of the main technical obstacles is the in vitro culturing of tumor cells of every single patient. The performance of gene transfer into tumor cells cultured for a relatively

short time period requires extraordinary experimental skills and is successful only in a portion of the cases. To date, infection with recombinant retroviruses represents the technique of choice for a gene transfer into this kind of cells.

EBV, EBV-derived Vectors, and EBV-immortalized Cells

EBV is present in lymphoblastoid cell lines (LCLs) in a state of latency. That means only a very small percentage of the infected cells produces infectious virus. In the state of latency, only six nuclear localized proteins (EBNA1, 2, 3A, B, C, LP) and two membrane-bound proteins (LMP and TP) of the virus are expressed. Generally, the EBV genome is present in the infected cell in episomal form in 10 to 100 copies. In the state of latency, the replication of the viral genome starts at an origin of replication (orip) (Yates et al., 1984). Maintenance of the episomal replication further requires binding of the EBNA1 protein to the oriP (Yates et al., 1984). The EBV-derived vectors consist of pBR sequences, oriP, an EBNA-1 expression cassette, and a selection marker specific for eukaryotic cells (e. g. the hygromycin resistance gene). Furthermore, these vectors have the capacity for additional 20 to 30 kb of foreign sequences. Constructs based on these vectors (i) show very much better "retention" in the cell even in the absence of selection (Middleton and Sugden, 1994); (ii) allow controlled expression without positional effects, and (iii) bear a substantially decreased risk of insertional mutagenesis. The efficiency of these vectors compared to mere plasmid vectors in obtaining stably transfected cells is substantially higher.

More than 95% of adult humans are infected by EBV. The primary infection occurs either asymptomatically or in the form of an infectious mononucleosis. The immunological control of the virus-infected cells in vivo has been very well investigated. The various latent gene products of the virus are recognized by specific cytotoxic T cells. Only in the state of extreme immunosuppression, e. g. as observed with AIDS patients or iatrogenically induced in transplant recipients, is there the possibility of polyclonally proliferating EBV-positive cells.

Enhancement of Gene Expression by Immunoglobulin Regulatory Elements

The synergistic way of function of the three regulatory elements (κMAR, κEi, κE3') of the immunoglobulin κ locus has first been demonstrated in the case of activation of the c-myc promoters P1 and P2 (Polack et al., 1993; Hörtnagel et al., 1995). In a chromosomal translocation observed in Burkitt lymphoma (BL) a co-localisation of the c-myc gene and the region of the human immunoglobulin κ locus located 3' of the "κ joining region" occurs, by which the c-myc gene is activated. A characteristic of this activation is an alteration in the usage of the promoters of the c-myc gene: the P1 promoter is used preferentially over the P2 promoter. In contrast, in normal non-transformed cells the P2 promoter is used preferentially. Furthermore, derepression of the c-myc gene on the level of transcriptional elongation is observed.

To study the mechanism of c-myc activation by t(2;8) translocation the interaction of the c-myc gene with several regions of the Igκ locus was examined. As a technique the stable infection of BL cells by episomally replicating EBV derived vectors has been selected. Into these vectors the c-myc gene was cloned under the control of two regions of the IgK locus. One of these regions extends from the J region up to about 1,2 kb 3' from the constant region (Cκ) while the second region encompasses the κ3'E located 12 kb 3' of Cκ. Measurement of the c-myc expression obtained by this construct and by several shortened forms (deletions) resulted first in the observation that these regions are necessary and sufficient for the BL-specific activation of the c-myc gene (Polack et al., 1993). By further deletions the responsibility of three elements of the Igκ locus, κMAR, κEi, and κE3', for the activation of the c-myc gene could be demonstrated (Hörtnagel et al, 1995). Furthermore, the chromatin structure of the contructs stably introduced into BL cells was examined by DNaseI mapping of hypersensitive sites (HSS). The typical HSSs were formed in the 5' region of the c-myc gene as well as in portions of the Igκ locus. This demonstrates the formation of a normal chromatin structure on the extrachromosomally replicating constructs.

Utilization of the Igκ elements in association with an episomal vector shows:

- a synergistic activation of a heterologous promoter (c-myc) by the Igκ elements;
- a large capacity of the episomal vectors;
- the formation of a regular chromatin structure.

The constructs described in the publication by Hörtnagel et al., 1995, were deletion constructs for the determination of regions of Ig which are essentially necessary for c-myc activation. It does not suggest the gene constructs according to the present invention containing the elements in a functional arrangement, and the use of the gene constructs according to the present invention.

One problem of the present invention to provide a gene construct for gene therapy avoiding the aforementioned disadvantages known from the state of the art.

This aim is achieved according to the invention by a gene construct containing, in functional association, at least:

(a)
- (i) a combination of two enhancer elements of the immunoglobulin κ locus, namely the κ intron enhancer (κEi) and the κ 3' enhancer (κE3'); or
- (ii) a combination of two enhancer elements of the immunoglobulin heavy chain μ locus, namely μEi and the μE3' enhancer region located 3' of Cα; or
- (iii) a combination of one or more of these enhancer elements of (ii) together with one or more of the aforementioned elements of the immunoglobulin κ locus; or
- (iv) the single enhancer element of the immunoglobulin λ locus; or
- (v) a combination of this enhancer element of (iv) together with one or more of the aforementioned elements of the immunoglobulin κ locus; or
- (vi) a combination of this enhancer element of (iv) together with one or more of the above elements of the immunoglobulin heavy chain μ locus; and further

(b) a promoter;

(c) a gene of interest, selected from at least one element of the group, consisting of cytokine gene, viral antigen, cellular adhesion gene, tumor antigen, co-stimulatory signalling molecule gene, a HLA gene non-identical with the recipient, and β-galactosidase gene; and

(d) a polyadenylation site (PAA).

Preferably, the gene construct according to the present invention contains the following combinations of regulatory elements:

κEi and λE, μEi and κE3', μEi and μE3', κEi and μE3', μEi and λE, κMAR and κEi and κE3', λE and κE3', λE and μE3'.

In a further preferred embodiment the gene construct of the present invention contains the immunoglobulin κ matrix attachment region (κMAR) or the immunoglobulin μ matrix attachment region (μMAR) in addition to one or more of the aforementioned enhancer elements or the combinations of enhancer elements.

The gene construct of the present invention is useful for gene therapy of diseases of the B cell system.

Preferably, the gene of interest may be the B7-1 or the B7-2 gene.

The gene construct of the present invention preferably contains sequences derived from EBV vectors, mini EBV vectors, bacterial vectors, from retroviruses, from adenovirus-associated viruses, from adenoviruses, or from vaccinia viruses. Further, it preferably contains sequences derived from bacterial vectors. In one embodiment of the invention, the sequence derived from EBV vectors or mini EBV vectors is the origin of replication (oriP).

In a preferred embodiment, the gene construct of the present invention additionally contains the EBNA1 expression cassette. Further, the gene construct of the present invention may additionally contain a marker gene, preferably being a resistance gene. This resistance gene is selected from resistance genes known as such. For example, an ampicillin resistance gene or a hygromycin resistance gene or a neomycin resistance gene may be employed.

The bacterial vector sequence may be chosen from any vector sequence known as such. Preferably, it is derived from pBR vectors.

In a particularly preferred embodiment, the gene construct of the present invention comprises EBV-derived vector sequences with or without the EBNA1 gene. Reference is made here to the complete contents of the publication of Sugden et al., 1985, and it shall be incorporated into this application for the completeness of the disclosure.

The promoter may be selected from any promoter capable of expressing the gene of interest in a selected cell. Preferably, the promoters of the group of a cell specific promoters, cytomegalovirus (CMV) promoter, tk and β globin promoters may be used.

The polyadenylation site may be derived from those sequences generally employed as a polyadenylation site. A representative polyadenylation site is the human β globin gene polyadenylation site or the SV 40 polyoma virus polyadenylation site.

The gene construct of the present invention allows the expression of a gene including therapeutically useful genes in a host cell. The protein encoded by the gene can then be produced by culturing the host cell under conditions that allow expression of the gene. Preferably, these therapeutically useful genes are expressed in B cells, B cell-derived cells, such as B cell tumor cells or cells immortalized by EBV or mini EBV, following successful gene transfer for the purpose of therapy of malignant and viral diseases.

Examples of the genes which may be expressed by the gene construct according to the invention are the cytokine genes, selected from the group of IL-2, -4, -6, -7, -8, -10, GM-CSF, G-CSF, TNF alpha, MCP 1, interferon gamma.

In a further embodiment of the present invention, into the construct of the present invention are inserted as a gene of interest a tumor antigen selected from the group of antigens members of human papilloma virus (HPV), melanoma-associated antigens, of the MAGE, BAGE, and GAGE gene family, the gp100, the idiotypes of T cell or B cell receptors of tumors, and mutated oncogenes. Particularly, the tumor antigens may be tumor-associated or tumor-specific antigens.

Preferably, the aforementioned tumor antigens are mutated oncogenes, such as ras genes or p53 genes or the derivatives thereof. The antigen associated with malignant melanoma is preferably the tyrosinase gene. Further genes of interest useful in the present invention are well-known to one skilled in the art and may be selected dependent on the disease to be treated.

The present invention also comprises such prokaryotic cells and eukaryotic cells transfected by one of the gene constructs of the present invention so as to contain the gene construct in an integrated or episomal, i. e. non-integrated, form. Preferably, the prokaryotic cell employed is an *E. coli* cell, and the eukaryotic cell employed is a B cell immortalized by EBV or mini EBV.

Preferably, the gene construct according to the present invention may contain as a gene of interest the viral antigens of HIV, CMV, HTLV1, and HPV, wherein said antigens are capable of inducing an immune reaction against the virus-infected cell.

The gene constructs according to the -invention may be used in the form of a pharmaceutical preparation further containing conventional carriers and/or excipients well known as such.

Preferably, the gene constructs of the invention are present in the pharmaceutical preparation according to the invention in liposomes or liposome-like structures.

In a further embodiment of the invention, the gene construct of the invention lacks a c-myc tumor antigen.

In a preferred embodiment of the invention there is provided a pharmaceutical preparation containing the gene construct of the invention in an effective amount together with conventional carriers and/or excipients.

In a further preferred embodiment of the present invention there is provided a pharmaceutical preparation containing primary B lymphocytes or fibroblast cells immortalized by EBV or mini EBV and comprising the gene construct of the present invention in an effective amount together with conventional carriers and/or excipients.

In a further preferred embodiment there is provided a pharmaceutical preparation containing primary B lymphocytes or fibroblast cells immortalized by EBV or mini 22V and comprising the gene construct of the present invention together with autologous T cells in an effective amount and conventional carriers and/or excipients.

In a further preferred embodiment there is provided a pharmaceutical preparation containing autologous T cells stimulated and expanded ex vivo using primary B lymphocytes or fibroblasts immortalized by EBV-or mini EBV and comprising the gene construct according to the invention.

By using an EBV-derived vector in combination with three specific regulatory elements of the immunoglobulin kappa locus, namely the matrix attachment region, the intron enhancer, and the 3' enhancer, a therapeutically beneficial gene can be specifically expressed in B cells over a prolonged time period. A further advantage of the use of the gene constructs of the invention is the ommission of the culturing of primary B cells, which is very complicated experimentally since immortalized cells (LCL cells) of almost any individual can be obtained by infection of peripheral B cells with EBV or mini EBV (see review in: Rogers et al., 1992). The establishment of these LCLs is much less complicated experimentally than the culture of primary tumor cells.

In the following the present invention will be explained in more detail refering to the attached Figures. Among the Figures :

FIG. 1: represents the construction and the structure of a gene construct according to the invention;

FIG. 2: represents schematically the cloning of one of the genes to be expressed into vector BC230 or into a vector derived therefrom;

FIG. 3: represents the expression of β-galactosidase in Burkitt's lymphoma (BL) cells transfected with DZ17 and CMV β-gal, respectively.

Preferably, the gene constructs according to the invention are based on vectors derived from Epstein Barr virus and containing the sequences for oriP, the EBNA1 expression cassette, resistance markers and, optionally, further pBR sequences.

Preferably, the vectors according to the present invention further contain a combination of different regulatory elements of the immunoglobulin kappa locus: the matrix attachment region (MAR), and the enhancer elements located in the intron (Ei) of the kappa locus as well as 12 kb 3' (E3') of the Ig kappa gene constant region.

Preferably, the promoter region of the expression cassette is derived from human cytomegalovirus (immediate early gene; CMV-P). Preferably, the polyadenylation site is derived from the human β globin gene. Further useful promoters are minimal promoters, such as the tk or the β globin promoter.

In a preferred embodiment the activity of the vector shall be restricted to B cells in order to meet safety requirements. For example, this can be achieved by introduction of the c-myc P1 promoter instead of the CMV promoter. An almost complete lack of activity of this promoter in the absence of enhancer could be demonstrated. Because of this property, this promoter appears to be particularly useful to achieve the restriction of the activity of the vector to B cells by using a B cell specific enhancer.

The sequence of the EBNA1 expression cassette is known. This expression cassette may be cloned into an EBV derived vector, as for example described by Yates et al., 1985. Particularly useful is the region between the oriP and the hygromycin resistance gene cassette (HYG).

The vectors of the present invention may carry as a reporter gene either the β galactosidase gene or the chloramphenicol acetyl transferase gene, and said gene may be under the control of the CMV promoter. Vectors useful for the transfection of EBV negative cells are such vectors carrying the expression cassette for the EBNA1 gene.

Different cytokine genes or tumor antigens may be cloned into the cloning sites from SfiI 842 through SfiI 862 or NaeI at 850. Cytokine genes useful in the present invention are for example: IL-2, -4, -6, -7, -8, -10, GM-CSF, G-CSF, TNF alpha, MCP 1, interferon gamma.

The viral antigens or tumor antigens which may be used include: viral antigens of viruses associated with the formation of tumors, such as antigens of the human papilloma virus (HPV), antigens associated with malignant melanoma, such as tyrosinase, the MAGE, BAGE and GAGE gene family, and the gp100, the idiotypes of T cell receptors or B cell receptors of tumors derived from T cells or B cells, mutated oncogenes, such as the ras gene or the p53 gene, and the like. Further, the viral antigens which may be used include antigens of HIV and of CMV capable of inducing an immune reaction against the virus-infected cell.

For direct gene transfer or ex vivo gene transfer into tumor cells an HLA gene may also be used which is foreign to the recipient, such as the HLA B7 gene or the gene of the co-stimulatorily acting B7 molecule.

Referring to FIG. 1, the construction of one of gene constructs of the invention, namely of the vector BC230 is described in the following by way of example.

The construction of BC230 was performed starting from the Epstein Barr virus derived vector p201 (pHEBNA) described by Sugden et al. (1985). Together with portions of pBR322 and a hygromycin resistance gene expression cassette (HYG), this vector contains the origin of replication (orip) of EBV (sequence position 7333 up to 9109 of B95.8 EBV [=designation in the EMBL data bank] as well as a 2,6 kbp region (sequence position 107567 up to 110176 of B95-8) coding for the EBNA1 (Epstein Barr virus nuclear antigen 1) gene.

Then, between the oriP and the hygromycin resistance gene were cloned the promoter/enhancer region of human cytomegalovirus (sequence position 467 up to 1218 of HEHCMVP1 [=designation in the EMBL data bank]), the chloramphenicol acetyl transferase gene (CAT) (sequence position 452 to 1240 of BLCAT3DNA [=designation in the EMBL data bank]) and the polyadenylation site of the rabbit beta globin gene (sequence position 31393 to 32554 of OCBGLOOI [=designation in the EMBL data bank]).

Between the β globin polyadenylation signal and the hygromycin resistance gene were introduced part of the polyadenylation site of c-myc (sequence position 7800 to 8056 of HSMYCKOB [=designation in the EMBL data bank]), the matrix attachment region (κMAR), the adjacent intron enhancer (κEi), and the 3' enhancer (κE3') located 3' of the constant region of the human kappa gene. The κMAR element has been defined as a HindIII-EcoRr fragment (sequence position 3237 to 3447 of HSIGK1 [=designation in the EMBL data bank]), and the κEi has been defined as an EcoRI-EcoRI fragment (sequence position 3447 to 4153 of HSIGK1 [=designation in the EMBL data bank]) (H örtnagel et al., 1995). The κE3' is represented by a Sau3AI-Sau3AI fragment heing 1169 bp in length.

The CAT gene may be removed by restricting with restriction enzyme SfiI. The resulting ends of the vector fragment (BC230vec) are non-compatible and therefore are not capable of being ligated with each other. Therefore, treatment of the vector fragment with phosphatase (CIP; calf intestinal phosphatase) can be omitted. A target gene to be expressed being present as a "blunt end" fragment (either by digestion with suitable restriction enzymes [enzymes generating "hlunt ends"], or by digestion with restriction enzymes followed by treatment with Klenow polymerase to fill in protruding ends) may be cloned into BC230vec using ligation with two SfiI adapters (see FIG. 2). Because of the incompatibility of the protruding ends generated on one side of the adapter no undesired circular molecules are formed during ligation. A further advantage of this procedure is that the gene segment to be cloned may contain the recognition sequence of SfiI enzyme. Therefore, restriction of the gene segment to be cloned with SfiI prior to ligation as it is necessary in the preparation of a "classical" linker ligation is dispensable.

By modification of the BC230 vector the following variations were generated:

a. without MAR element: the HindIII-EcoRI fragment was removed (BC229);

without EBNA1 gene for expression in EBV positive cells (BC243);

inclusion of the β galactosidase gene instead of the CAT gene (DZ17);

the genes which may be cloned into the BC230 vector include for example the following : IL-2, -4, -6, -8, -10, GM-CSF, B7-1, IFN γ, TNF α; those vectors are referred to as BC219.

EXAMPLE 1

In the FIG. 3 the advantages of the utilization of the vector of the invention are exemplified by DZ17 containing the β galactosidase gene.

BL cells (Raji) were transfected transiently by electroporation with DZ17 vector and the CMV β-gal construct (lacking the enhancer cassette and portions of EBV; β-gal=β galactosidase). Afterwards, the cells were cultered for 16 days in the absence of hygromycin (i. e. in the absence of selection). After 2, 7, 12, and 16 days aliquots of the cells were recovered and the expression of β galactosidase was determined. FIG. 3 shows the results of this assay. While, after two days the expression of the two plasmids differed by about a factor of 4, a significant difference was observed already after 7 days. After 16 days, no expression of β galactosidase in the cells transfected by CMV β-gal could be detected. In cells transfected by DZ17, a maintenance of β galactosidase activity could be detected which was 10 times lower but still high. This result has to be interpreted in a way that the vector of the invention fulfils the property of being retained in cells achieving a high expression and ensuring the expression over a prolonged time period.

EXAMPLE 2

Efficiency of Transfection

An EBV-positive (BL60) and an EBV-negative (BJAB) human lymphoma cell line were chosen as a target cell for the comparison of a conventional integrating vector (pKEX, Rittner et al., 1991) and vector BC219, a derivative of BC230 lacking the CAT gene. BC219 was used instead of BC230 because BC230 expresses chloramphenicol acetyl transferase (CAT). High doses of this enzyme in the cell may exert toxic activity and thereby complicate the comparison. pKEX contains the CMV promoter/enhancer region, a SV40-derived polyadenylation site, the hygromycin resistance gene expression cassette, and a portion of pBR including the origin of replication for multiplication in bacteria. This vector as well as its derivatives have to be integrated into the cellular genome for stable replication within the target cells.

DNA from BC219 and pKEX was introduced into both cell lines by electroporation (method described by Polack et al., 1991). After 24 h in the absence of selection pressure, the cells were seeded at a density of 2×10^5 cells/ml into a 96-well cell culture plate in a volume of 100 μl/well under selective conditions (400 μg/ml hygromycin). The samples showing cellular proliferation were counted, grown, and tested further. The following table shows the results of an experiment of the aforementioned type

TABLE 1

Cell line	Vector	Transfection efficiency Growth per 1×10^6 cells plated
BJAB	pKEX	10
	BC219	100
BL60	pKEX	0.3
	BC219	500

This result can be explained in a way that stable transfectants can be prepared much more readily using the BC230 derivative BC219.

EXAMPLE 3

Cytokine Expression using BC230 Derivatives

As shown in the FIG. 1, the cDNAs of TNFα, GM-CSF and IL6 were cloned into vector BC230 and into its derivative BC219, and into pKEX, respectively. Using these constructs, different transformants were generated after stable transfection and selection with hygromycin. The production of the different cytokines was determined in the cellular supernatant of the different transfectants using a commercially avaible ELISA (Biermann Co., Bad Nauheim, Germany). The BL60 cells fail to produce any of these cytokines either spontaneously or after transfer of BC230 or pKEX. In any of these cases, transfectants generated by the integrating vectors (pKEX and derivatives) failed to produce a detectable amount of TNFα, GM-CSF or IL6, respectively.

All of the ten different sublines generated by each of the three cytokine vectors based on vector BC219 secreted about the same amount of cytokine into the supernatant. The variation in the cytokin production of the individual transfectants is listed in the following table:

TABLE 2

Cell line	Construct	Number of clones analysed	Cytokine production (variation)
BL60	pKEX-CAT	10	not detectable
	pKEX-IL6	5	n. d.
	pKEX-TNF α	9	n. d.
	BC230	10	n. d.
	BC219-TNF α	10	1500-2000 pg/ml TNF α
	BC219GM-CSF	10	700 pg/ml GM-CSF
	BC219-IL6	10	900-1000 pg/ml IL-6

After transfer either ex vivo or in vivo, EBV-derived vectors have the capability of being retained within cells in the absence of selection. Therefore, expression of genes transferred by these vectors occurs over a prolonged time period. Thus, the direct liposome-mediated intratumour transfer of a construct expressing for example GM-CSF, e. g. into a B cell lymphoma in order to stimulate the body's own defenses against the tumor cells, is an effective therapeutic.

EBV Immortalized B Cells as Target Cells for Gene Transfer

In vitro culturing of tumor cells is only successful for a part of the tumor patients. By using EBV, immortalized B cells of any individual can be produced in unlimited amounts. These cells can be made to produce messenger substances (cytokines) effective in immunomodulation by gene transfer with the aforementioned construct. To stimulate an immune reaction directed against a tumor, the cells generated in this manner can be inoculated, e. g. subcutaneously, directly into the tumor as well as in combination with tumor cells. For safety, these cells may be prevented from further proliferation by irradiation prior to transplantation.

Further, EBV immortalized cells may be made to express viral or tumor antigens by transfection of the corresponding gene constructs. These antigens should be presented by the cells in an MHC restricted manner. Therefore, those cells are useful for the stimulation of autologous T cells recognizing these antigens. The thereby stimulated T cells may be expanded using those antigen-presenting cells and IL-2. After transfer, the expanded T cells in vivo will specifically attack the virally infected cells or the tumor cells expressing the antigen.

Using EBV establishment of cell lines from any individual becomes relatively simple. These cells can be provided ex vivo with gene constructs and then retransplanted into the syngenic individual. In an adoptive immune therapy, the cells may be employed either as producers of cytokines

or as antigen-presenting cells. In all embodiments, EBV may also be replaced by mini EBV.

Thus, in conclusion, a vector is. provided preferably based on EBV which allows the achievement of a high and persistant expression, i. e. a stable expression, in cells, particularly in B cells, which expression is an improvement over all other known vector systems. Genes of therapeutical value such as cytokine genes or tumor antigens can be stably incorporated into the B cells to be treated and used in the therapy of malignant and viral diseases of man, in particular of B cell tumor-associated diseases. All publications refrred to herein are hereby expressly incorporated by reference in their entirety. The gene constructs of the present invention are especially useful for the use in gene therapy.

What is claimed is:

1. A gene construct for the expression of a polypeptide, comprising:

(a) an enhancer comprising an element or combination of elements, wherein said element or combination of elements is selected from the group consisting of:

(i) a combination comprising the following elements obtained from the immunoglobulin κ locus: the κ intron enhancer (κEi), the κ 3' enhancer (κE3'), and the immunoglobulin κ matrix attachment region (κMAR);

(ii) a combination comprising the following elements obtained from the immunoglobulin heavy chain μ locus: the μ0 intron enhancer (μEi) and the μ 3' enhancer obtained from a region located 3' of Cα (μE3');

(iii) a combination of

one or more elements selected from the group consisting of κEi and κE3+ and

one or more elements selected from the group consisting of μEi and μE3';

(iv) the enhancer element of the immunoglobulin λ locus (λE);

(v) a combination of

λE and

one or more elements selected from the group consisting of κEi and κE3'; and

(vi) a combination of

λE and

one or more elements selected from the group consisting of μEi and μE3';

(b) a promoter operably linked to said enhancer;

(c) a nucleotide sequence operably liked to said promoter, said nucleotide sequence encoding a polypeptide selected from the group consisting of: a cytokine, a viral antigen, a cellular adhesion protein, a tumor antigen with the proviso that the c-myc-antigen is excluded, a co-stimulatory signalling molecule, an HLA antigen, and β-galactosidase; and

(d) a polyadenylation site (PAA) operably linked to said nucleotide sequence.

2. The gene construct according to claim **1**, wherein said enhancer comprises a combination of enhancer elements selected from the group consisting of:

κEi and λE, μEi and κE3', μEi and μE3', κEi and μE3', μEi and λE, the immunoglobulin κ matrix attachment region (κMAR) and κEi and κE3', λE and κE3', and λE and μE3'.

3. The gene construct according to claim **1**, wherein said gene construct additionally comprises the immunoglobulin μ matrix attachment region (μMAR).

4. The gene construct according to claim **1**, wherein said polypeptide is an HLA antigen.

5. The gene construct according to claim **4**, wherein said HLA antigen is HLA B7.

6. The gene construct according to claim **1**, wherein said gene construct further comprises an additional sequence obtained from a source selected from the group consisting of an EBV vector, a mini EBV vector, a bacterial vector, a retrovirus, an adenovirus-associated virus, an adenovirus and a vaccinia virus.

7. The gene construct according to claim **6**, wherein said additional sequence is derived from an EBV vector and comprises the origin of replication of EBV (oriP) and a resistance gene said gene construct additionally comprising a sequence derived from a pBR vector.

8. A gene construct according to claim **7**, additionally comprising an expression cassette for EBNA1.

9. The gene construct according to claim **6**, wherein said additional sequence is selected from the group consisting of:

(a) a sequence derived from an EBV vector consisting of the origin of replication (oriP), a sequence encoding EBNA1, and a sequence encoding a marker gene; and

(b) a sequence derived from a pBR vector.

10. The gene construct according to claim **1**, wherein said gene construct further comprises a marker gene.

11. The gene construct according to claim **10**, wherein said marker gene is a resistance gene selected from the group consisting of an ampicillin resistance gene, a hygromycin resistance gene, and a neomycin resistance gene.

12. The gene construct according to claim **1**, wherein said promoter is selected from the group consisting of a B cell-specific promoter, a cytomegalovirus (CMV) promoter, a tk promoter, a β globin promoter, and a c-myc P1 promoter.

13. The gene construct according to claim **1**, wherein said polyadenylation site is derived from a source selected from the group consisting of a human β globin gene or an SV40 polyomavirus.

14. The gene construct according to claim **1**, wherein said polypeptide is a cytokine selected from the group consisting of IL-2, -4, -6, -7, -8, -10, GM-CSF, G-CSF, TNT alpha, MCP 1, and interferon gamma.

15. The gene construct according to claim **1**, wherein said polypeptide is a viral antigen selected from the group consisting of an HIV antigen, an HTLV1 antigen, an BPV antigen, and a CMV antigen.

16. A composition comprising a liposome or a liposome-like structure and the gene construct according to claim **1**.

17. An isolated cell comprising the gene construct of claim **1**.

18. The isolated cell according to claim **17**, wherein said cell is an *E. coli* cell.

19. The isolated cell according to claim **17**, wherein said cell is a eukaryotic cell.

20. The isolated cell of claim **19** wherein the gene construct is present in said cell in an integrated form in a chromosome of said cell.

21. The isolated cell of claim **19**, wherein the gene construct is present in said cell in an episomal form.

22. A process for producing the polypeptide encoded by said gene construct, comprising culturing the cell of claim **21** under conditions whereby said polypeptide is produced by said cell.

23. The isolated cell of claim **19**, wherein the cell is selected from the group consisting of a B cell and a fibroblast cell and wherein said cell is immortalized by EBV or mini EBV.

24. A process for producing the polypeptide encoded by said gene construct comprising culturing the cell of claim **17** under conditions whereby said polypeptide is produced by said cell.

25. The process of claim 24, wherein said cell is a eukaryotic cell.

26. A process for stimulating T cell growth in culture, comprising contacting a T cell with an immortalized cell immortalized by EBV or mini EBV, said immortalized cell selected from a primary B lymphocyte and a fibroblast, said immortalized cell comprising a gene construct according to claim 1, wherein said contacting is cared out for a time sufficient to stimulate the growth of said T cell.

27. The process of claim 26 wherein said gene construct was introduced into said immortalized cell by gene transfer.

28. A process for producing an insulated cell comprising a gene construct, said process comprising introducing the gene construct of claim 1 into said cell by gene transfer, wherein said cell is a primary B lymphocyte or a fibroblast, wherein said cell is immortalized by EBV or mini-EBV.

29. The gene construct according to claim 1, wherein said polypeptide is a co-stimulatory signaling molecule.

30. The gene construct according to claim 29, wherein said co-stimulatory signaling molecule is encoded by a gene selected from a B7-1 gene and a B7-2 gene.

31. The gene construct according to claim 1, wherein said polypeptide is a tumor antigen.

32. The gene construct according to claim 31, wherein said tumor antigen is selected from the group consisting of a tumor-associated antigen and a tumor-specific antigen.

33. The gene construct according to claim 31, wherein said tumor antigen is encoded by an oncogene.

34. The gene construct according to claim 31, wherein said tumor antigen is selected from the group consisting of ras, p53, derivatives of ras, derivatives of p53, and tyrosinase.

35. The gene construct according to claim 31, wherein said tumor antigen is selected from the group consisting of an antigen of the human papilloma virus (BPV), a malignant melanoma-associated antigen, a MAGE antigen, a BAGE antigen, a GAGE antigen, gp100, a tumor idiotype of a T cell receptor, a tumor idiotype of a B cell receptor, and a tumor antigen encoded by a mutated oncogene.

36. A process or producing a cell comprising a gene construct, said process comprising:

(a) immortalizing a primary B lymphocyte to produce an immortalized B lymphocyte; and

(b) introducing the gene construct according to claim 1 into said immortalized B lymphocyte.

* * * * *

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Suitability of Epstein–Barr virus-based episomal vectors for expression of cytokine genes in human lymphoma cells

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Plasmids carrying the Epstein–Barr virus (EBV) latent gene EBNA1 and the EBV latent origin of replication (oriP) stay in transfected human cells as autonomously replicating extrachromosomal genetic units. They thus might represent a suitable tool for cytokine gene introduction into human tumor cells with the prospect of therapeutic anti-tumor vaccination. The aim of this study was to analyze whether such plasmids permit stable and efficient expression of cytokine genes in human non-Hodgkin lymphoma cells. We tested physical stability and expression levels of plasmids carrying EBNA1 and oriP for episomal maintenance, immunoglobulin light chain enhancer elements for augmentation of expression, and cytokine or marker genes after introduction into human NHL cell lines in vitro and in vivo after inoculation into nude mice. Data obtained with these EBV-based vectors were compared with another plasmid, not carrying EBNA1 and oriP. cDNAs coding for GM-CSF, IL6, TNFα, the chloramphenicolacetyl-transferase (CAT) and the β-galactosidase (lacZ) gene were transfected into the EBV-positive Burkitt's lymphoma cell line BL60 and the EBV-negative B cell lymphoma cell line BJA-B. EBV-derived vectors permitted a high, host cell independent transfection efficiency and high and host cell independent levels of expression. After removal of the selection pressure (hygromycin B) cytokine expression could be detected for several weeks in vitro and in vivo but, however, declined continuously. These experiments suggest that episomal BC-derived vectors represent an effective tool for cytokine gene transfer in human lymphoma cells.

Keywords: *cytokine gene transfection; episomally replicating plasmids; EBV latent origin of replication; malignant lymphoma*

Introduction

Induction of an antitumor host response by vaccination with genetically modified, cytokine secreting autologous tumor cells has been demonstrated in several murine tumor models. In these studies mice were vaccinated, for instance, with interferon gamma (IFNγ)-transduced neuroblastoma cells,[1] interleukin 2 (IL2)-transduced fibrosarcoma cells[2] or IL2-transduced colon carcinoma cells.[3] Other vaccination approaches included transduction of malignant cells with cDNA coding for granulocyte–macrophage colony-stimulating factor (GM-CSF,, IL4, IL7, IL8 or tumor necrosis factor alpha (TNFα).[4] Cytokine expression in tumor cells was shown to activate MHC-restricted cytotoxic T cells, lymphokine-activated killer (LAK) cells, natural killer (NK) cells and macrophages.[5] These promising animal studies have now initiated several clinical phase I/II trials using solid tumor cells transduced with retroviral constructs expressing human cytokine genes.[6]

Malignant lymphomas also represent attractive candidates for cytokine transfer-mediated immune therapy. Immunoglobulin (Ig) gene idiotypes expressed on B lymphoma cells are tumor-specific antigens and thus represent individual targets for specific immune therapy. In mice a humoral as well as a cellular immune response have been demonstrated against Ig idiotypes. Furthermore, idiotype immunization led to tumor regression of murine B cell lymphoma.[7] This effect could be augmented by systemic administration of IL2 and IFNγ.[8] In the murine model the idiotypes themselves have only a weak immunogenic potential. A pronounced augmentation of idiotype immunogenicity, was achieved by use of idiotype/GM-CSF fusion proteins.[9] Yet, no data exist with regard to augmentation of an immune response following cytokine gene transfection into human lymphoma cells.

The most widely used gene transfer systems are replication-incompetent retroviruses permitting a high transduction efficiency. Retroviral gene transfer, however, involves some still unsolved problems. Owing to integration of the vector into the host cell genome, expression

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of transduced genes may vary with the integration site and cell type. In addition, insertional mutagenesis represents a possible hazard.[10] Thus, alternative viral and nonviral gene delivery and expression systems are being explored.

Recently, infectious EBV-based vectors (mini-EBVs) were engineered to transfer genes into human B cells.[11] These infectious mini-EBVs carried *cis*-acting elements of EBV necessary for episomal maintenance, amplification and packaging of the vector, not however, any of the known latent EBV genes associated with transformation. Successful infection of B lymphoblastoid cells from a Fanconi anemia group C (FA-C) patient with a mini-EBV vector expressing the normal FACC cDNA could be demonstrated. Compared with retrovirus-mediated gene transfer, these mini-EBVs had the advantage of replicating episomally in the target cells, thus avoiding the possible hazards of integration into the host cell genome. However, there might still be concerns about the incalculable hazards of working with infectious particles. This concern might especially hold true for the cloning of cytokine genes into such vectors, which then might exert pleiotropic growth promoting and growth inhibiting effects.

For *ex vivo* cytokine transfer in lymphoma (and other tumor) cells it would be desirable to use potent expression vectors, which have the advantage of episomal replication, but do not represent infectious particles. In this study we have tested cytokine gene introduction and expression in human lymphoma cell lines using plasmids containing the Epstein–Barr virus nuclear antigen 1 (EBNA1) gene and the viral latent origin of replication (oriP) permitting the episomal state of these plasmids and in addition immunoglobulin kappa light chain enhancer elements for augmentation of expression. Results with these episomal vectors were systematically compared with results obtained with a vector not carrying these EBV and lg kappa light chain gene-derived genomic elements.

Results

Construction of expression vectors

The expression vectors pKEX-CAT, pKEX-TNFα and pKEX-IL6 were obtained by cloning cDNAs for the *CAT* gene, TNFα and IL6 into the pKEX-2XR-plasmid. The expression vectors BC219-IL6, BC219-TNFα and BC219-GM-CSF were obtained by cloning the cDNAs into the plasmid BC219, which replicates episomally after transfection due to the presence of the EBV nuclear antigen 1 (EBNA1) and the EBV latent origin of replication (oriP). Table 1 summarizes the vectors constructed. Figure 1 shows the maps of the basic vectors. For detailed cloning strategy see Materials and methods.

Table 1 Vectors constructed for *CAT*, *lacZ* and cytokine expression

Nonepisomal vectors (pKEX-2XR-derived)[a]	*Episomal vectors (BC219-derived)*[a]
pKEX-CAT	BC230a (=BC219-CAT)
pKEX-IL6	BC219-IL6
pKEX-TNFα	BC219-TNFα
	BC219-GM-CSF
	BC219-DZ17 (=*lacZ*)

[a]For detailed map see Figure 1a and b.

Transfection of BL60-P7 and BJA-B cells with pKEX-2XR- and BC219-derived expression vectors

pKEX-CAT, pKEX-IL6 and pKEX-TNFα were transfected into the Burkitt's lymphoma cell line BL60-P7 and the EBV-negative B cell lymphoma cell line BJA-B using electroporation. The number of growing, hygromycin B-resistant clones was determined 3 weeks after electroporation. The stable transfection efficiency was defined as the ratio of hygromycin B-resistant clones to viable cells after electroporation. For each cell line and vector type transfection efficiencies were rather similiar, regardless of the given insert. Using the pKEX constructs, average transfection efficiency was approximately 1 to 7.1×10^4 for BJA-B cells and 1 to 2.5×10^6 for BL60-P7 cells.

The BC219-IL6, BC219-TNFα, BC219-GM-CSF, BC230a (=BC219-CAT) and BC-DZ17 (=BC219-*lacZ*) were transfected into BL60-P7 and BJA-B cells by the same procedure. For these vectors the average transfection efficiency was approximately 1 to 2.4×10^3 for BJA-B cells and 1 to 2.7×10^4 for BL60-P7 cells. Thus, by comparison with pKEX-2XR, BC219-derived vectors show a 3.2-fold higher transfection efficiency in BJA-B cells and a 92-fold higher transfection efficiency in BL60-P7 cells (see also Table 2).

Southern blotting was performed to determine the physical state of the transfected pKEX-plasmids in hygromycin B-resistant BL60-P7 and BJA-B transfectants. Cellular DNA from four representative BL60-pKEX-CAT transfectants was digested with *Bam*HI and hybridized with the ^{32}P-labeled pKEX-CAT DNA (Figure 2a). *Bam*HI digestion of pKEX-CAT plasmid results in two fragments of 6.0 and 1.6 kb length, respectively. Thus, after integration of the pKEX-CAT plasmid into the BL60 genome in Southern blot analysis, three *Bam*HI fragments are expected. The total length of these fragments should be at least 7.6 kb (plus the length of adjacent cellular fragments). In all four BL60-pKEX-CAT clones three fragments were detected. The upper band of 5.8 kb size is due to hybridization of the pKEX-CAT plasmid to the pSV2neo-plasmid[12] which had been transfected into this cell line previously.[13] The additional two bands which differ in size in all transfectants are interpreted to represent integrated pKEX-CAT sequences joined to BL60-P7 sequences. In all four clones the detected pKEX-CAT fragments are shorter than the total length of 7.6 kb of the pKEX-CAT plasmid. This may be due to a partial deletion of the plasmid during the integration process into the cellular genome. This might underlie the non-expression of CAT protein of these transfectants (see below) since promoter–CAT gene sequences could be affected by the deletion.

Southern blot analysis was also performed to determine the physical state of the transfected vectors in the BJA-B and BL60-P7-BC transfectants. Figure 2b shows DNA of three BJA-B-BC230a and three BL60-BC230a transfectants which were *Bam*HI digested and hybridized with the ^{32}P-labeled BC230a plasmid. In all BJA-B and BL60 clones exclusively the expected two fragments of 12.9 and 2.0 kb, respectively, were found to be generated by digestion of the BC230a plasmid with *Bam*HI demonstrating the episomal state of the vector. The additional band in all BL60-P7 clones of 5.8 kb is generated from

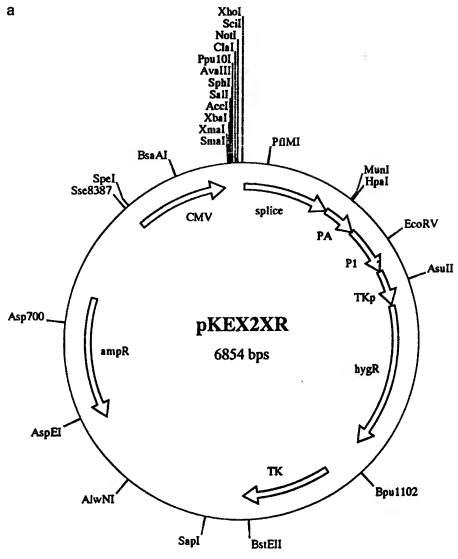

Figure 1 (a) Map of pKEX-2XR. CMV: human cytomegalovirus immediate-early promoter–enhancer element; splice and PA: simian virus 40 (SV40)-derived small T-antigen splice and late gene polyadenylation signal; TKp: thymidine kinase promoter; hygR: hygromycin B resistance gene; ampR: ampicillin resistance gene.

cross-hybridization of the BC230a probe to the pSV2neo-plasmid (see above). Figure 2c shows Southern blot analysis of four BJA-B and BL60-BC-TNFα transfectants which were hybridized with a ^{32}P-labeled BC219 probe. Again an identical hybridization pattern is found in all clones, namely two bands of 12.9 and 2.0 kb which represent the *Bam*HI digested BC-TNFα plasmid. The 5.8 kb fragment in the BL60-clones is again generated by cross-hybridization with the pSV2neo-plasmid.

Expression of cytokines and CAT protein in BJA-B and BL60-P7 transfectants

For each pKEX-2XR-derived expression vector, namely pKEX-CAT, pKEX-IL6 and pKEX-TNFα, 10 stable BL60-P7 and 10 stable BJA-B transfectants were isolated. After 24 h culture in fresh medium, the cytokine concentration of 1×10^6 exponentially growing cells/ml culture medium and CAT protein in 50 μg of total extracted cellular protein were measured. CAT expression of the BJA-B-pKEX-CAT clones reached from 90 to 2100 pg/ul. Cytokine concentration was measured from 8 to 700 pg/ml for the BJA-B-pKEX-IL6 and from 40 to 2050 pg/ml for the BJA-B-pKEX-TNFα clones. In contrast, no expression could be found in the BL60-P7 cells stably transfected with pKEX-2XR-derived expression vectors (Table 3). Cytokine and CAT expression was also measured for BL60-P7 and BJA-B clones stably transfected with BC219-derived vectors. In 10 out of 10 BJA-B and BL60-P7 clones, respectively, high expression levels for each transfected gene were found. IL6 expression ranged between 900 and 1210 pg/ml, TNFα expression between 1350 and 1900 pg/ml, GM-CSF expression between 650 and 655 pg/ml and CAT protein expression between 3800 and 5100 pg/ml (Table 3).

Taken together these data demonstrate that transfection with pKEX-2XR-derived expression vectors leads to quite variable expression patterns differing considerably between transfected clones of the same cell line as well as in between the cell lines BJA-B and BL60-P7, with no detectable expression in the latter. In contrast, transfection with the BC219 vector leads to transfectants with quite similiar expression levels in all BJA-B and BL60-P7

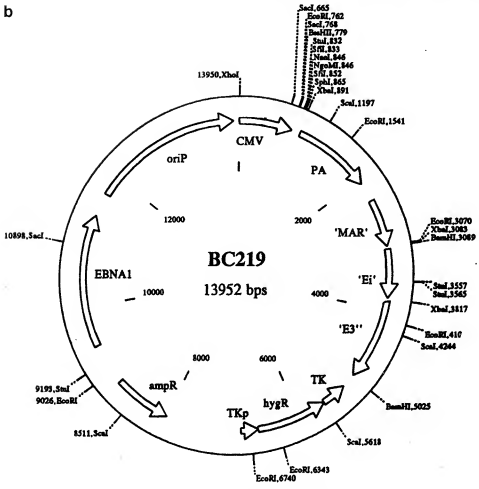

Figure 1 (b) Map of BC219. MAR: matrix attachment region; Ei: intron enhancer element; E3: 3'enhancer element of the human Ig kappa light chain gene locus; EBNA1: Epstein–Barr virus nuclear antigen 1 gene; oriP: Epstein–Barr virus latent origin of replication.

clones. In addition, the absolute amounts of cytokines or CAT protein were higher in all BJA-B and BL60-P7 clones transfected with BC219-derived vectors compared to pKEX-2XR-derived transfectants with the exception of BJA-B-pKEX-TNFα clones with expression levels comparable to those of BJA-B-BC-TNFα clones.

LacZ expression in BL60-P7 and BJA-B transfectants

To analyze the expression status of the episomal vectors in single cells, one clone of each BL60-P7 and BJA-B transfected with BC-DZ17, containing the *lacZ* gene, were stained for *lacZ* expression in a β-gal-*in situ* assay. Blue staining of the cells indicates expression of the *lacZ* gene. As shown in Figure 3a, about 50% of the BL60-BC-DZ17 clone 1.3.5. expressed the *lacZ* gene with a varying intensity. In BJA-B-BC-DZ17 clone 4.3.5. about 80% of the cells were positive for *lacZ* expression (Figure 3b). The number of positive cells was constant in both clones over a cultivation period of 6 months after transfection using hygromycin B selection.

No changes of endogenous cytokine expression in BL60-P7 cells after transfection with BC219-derived vectors

Introduction of foreign DNA into eukaryotic cells may influence their endogenous gene expression pattern. To analyze whether transfection of BL60-P7 cells with BC219 vectors leads to altered cytokine expression, BL60-BC219 transfectants were examined for their endogenous cytokine expression using ELISA. Untransfected BL60-P7 cells did not express the cytokines IL6, IL8, IL10 and TNFα. BL60-BC219 transfectant clones also did not express these cytokines (data not shown). Thus, transfection of BL60-P7 cells with BC219 vectors seems not to induce endogenous cytokine expression and the IL6 expression of BL60-BC-IL6 and TNFα expression of BL60-BC-TNFα transfectants, respectively, is due to expression of the transfected BC219-derived vector.

Stability of expression of pKEX-2XR- and BC219-derived expression vectors in vitro and in vivo

To analyze whether release of selection pressure leads to loss of the vectors transfected, one BJA-B-pKEX-TNFα clone and one BJA-B-pKEX-IL6 clone were cultivated without hygromycin B selection. TNFα and IL6 expression were measured over a period of 8 weeks in the supernatant of continuously proliferating cells. The BJA-B-pKEX-IL6 clone 1.8.6. expressed 250 pg/ml/24 h IL6 4 weeks after transfection. No significant decrease of expression could be found in the culture without hygromycin B after 36 weeks when IL6 expression was still 210 pg/ml/24 h. Similarly, TNFα expression of BJAB-pKEX-

Table 2 Transfection efficiencies of pKEX-2XR and BC219 plasmids after electroporation of BL60 P7 and BJA-B cells

Transfected cell line	*Viable cells seeded after electroporation*	*Number of stable transfected clones*	*Transfection efficiency*
BJA-B-pKEX-2XR	1.2×10^7	196	$1/6 \times 10^4$
BJA-B-pKEX-IL6	9.5×10^6	101	$1/9.3 \times 10^3$
BJA-B-pKEX-TNF	1.1×10^7	178	$1/6.1 \times 10^4$
BJA-B-pKEXCAT	7.5×10^6	107	$1/7.0 \times 10^4$
BL60-pKEX-2XR	9.8×10^6	3	$1/3.3 \times 10^6$
BL60-pKEX-IL6	5×10^6	2	$1/2.5 \times 10^6$
BL60-pKE X-TNF	7.5×10^6	3	$1/2.5 \times 10^6$
BL60-pKEX-CAT	9.6×10^6	5	$1/1.9 \times 10^6$
BJA-B-BC219	1×10^7	800	$1/1.3 \times 10^4$
BJA-B-BC-IL6	4.5×10^6	225	$1/2.0 \times 10^4$
BJA-B-BC-TNFα	8.4×10^6	210	$1/1.0 \times 10^4$
BJA-B-BC-GM-CSF	3.5×10^6	163	$1/2.1 \times 10^4$
BJA-B-BC230a	1.1×10^6	135	$1/8.1 \times 10^3$
BJA-B-BC-DZ17	5.4×10^6	432	$1/1.3 \times 10^4$
BL60-BC219	6.8×10^6	338	$1/2 \times 10^4$
BL60-BC-IL6	6.8×10^6	270	$1/2.5 \times 10^4$
BL60-BC-TNFα	1.3×10^7	990	$1/1.3 \times 10^4$
BL60-BC-GM-CSF	3×10^6	121	$1/2.4 \times 10^4$
BL60-BC230a	1.5×10^7	273	$1/5.5 \times 10^4$
BL60-BC-DZ17	6.5×10^6	258	$1/2.5 \times 10^4$

TNFα clone 2.2.5 did not change significantly after release of selection pressure. Taken together these data demonstrate a stable cytokine expression reflecting the stable integration of pKEX-2XR-derived expression vectors in BJA-B transfectants without hygromycin B selection.

The stability of cytokine expression of BL60-BC-IL6 transfectants was examined. One BL60-BC-IL6 clone was cultivated *in vitro* over a period of 9 weeks with and without hygromycin B selection and IL6 expression was measured weekly in the supernatant of continuously proliferating cells using ELISA. While IL6 expression remained stable in BL60-BC-IL6 cells cultivated with hygromycin B selection over the whole period, BL60-BC-IL6 transfectants cultivated without hygromycin B showed a decrease of IL6 expression. After 9 weeks cells expressed only 12.5 pg/ml IL6 in comparison to 456.5 pg/ml in the first week (Table 4). A similar IL6 expression pattern was found for BL60-BC-IL6 transfectants *in vivo*. Four nu/nu mice were inoculated with 1×10^7 BL60-BC-IL6 cells in each flank. After 2, 4 and 6 weeks, mice were killed, tumor cells were recultivated and IL6 expression was measured after 24 h. IL6 expression decreased from 212.6 pg/ml after 2 weeks to 94.3 pg/ml after 5½ weeks (Table 5). These data demonstrate that under *in vivo* conditions, without selection pressure, cytokine expression can be achieved only transiently with BC-derived expression vectors.

Discussion

The attraction of modified viruses for gene therapy is mainly based on their capacity to infect a high percentage of cells *in vitro* and *in vivo*. Thus, retroviruses, adenoviruses, adeno-associated viruses (AAV), herpesviruses and others have been modified as vectors for introduction of cDNAs in human tumor cells.[14–19] However, the use of infectious viruses in gene therapy has also been described to be associated with disadvantages, eg insertional mutagenesis inducing secondary cancers,[20] recombination events leading to pathogenic infectious agents,[21] induction of an immune response against the viral vectors[22] and, last but not least, the complexity of the manufacturing procedures.

Noninfectious, plasmid-based expression vectors may circumvent several of these problems. These vectors can be introduced into the target cells, for instance by direct inoculation of the DNA (naked DNA approach), after mixture with polycationic lipids (liposome-mediated gene delivery) or by complexing with polycations such as poly-(L-lysine).[23–27]

As a rule such plasmid vectors persist in the majority as episomal elements and permit short-term gene expression. A minority of the transfected plasmids, however, become integrated by chance into the host cell genome. In contrast, plasmids containing the EBV latent protein EBNA1 and in addition the viral latent origin of replication (oriP) are stably maintained after transfection as autonomously replicating episomes.[28] However, no data have been raised with regard to their long-term stability *in vivo*.

We were interested, whether such EBV-derived vectors might be suitable for cytokine gene expression in lymphoma cells with the prospect of vaccination therapy using cytokine-transfected lymphoma cells. Thus, we cloned the cDNAs for different human cytokines and for the marker genes CAT and *lacZ* into expression vectors carrying EBNA1, oriP and in addition human immunoglobulin kappa light chain enhancer elements (BC219-derived plasmids). After transfection into a human EBV-positive and an EBV-negative lymphoma cell line we tested stable transfection efficiency, expression level and

long-term expression *in vitro* and *in vivo* in a nude mouse model. The results were systematically compared with results obtained after transfection of plasmid vectors not carrying the EBV elements for episomal maintenance (pKEX-derived vectors)[29] into the same cell lines.

The episomal EBNA1/oriP vectors permitted in general a higher transfection efficiency compared with the pKEX-derived vectors, ie about a three-fold elevated number of stably transfected clones for cell line BJA-B and about 90-fold augmented for the BL60 cell line. These results reflected the stable, cell line-independent episomal maintenance of the EBNA1/oriP vectors during selection of hygromycin B-resistant clones after transfection. This was confirmed by Southern blot analysis which demonstrated the episomal state of the vectors without any evidence of integration into the host cell genome. By comparison, the lower rate of stable transfected cells using the pKEX-derived vectors reflected loss of episomal plasmid DNA shortly after transfection and the long-term persistence exclusively of vector DNA integrated into the host cell genome, as also demonstrated by Southern blot analysis.

Expression analysis after transfection of the EBNA1/oriP vectors revealed high cytokine concentrations in the supernatant of proliferating cells and high levels of intracellular CAT protein, respectively, in BL60 as well as in BJA-B cells. With the exception of TNFα, expression levels were higher with EBNA1/oriP vectors compared with pKEX-derived vectors. Moreover, and also in contrast to pKEX-derived vectors, with the EBNA1/oriP vectors expression levels of a given cytokine differed only slightly between different transfected clones of the same cell line as well as between the two cell lines tested. This observation confirmed the absence of position effects on gene expression due to vector integration when using the EBNA1/oriP vectors.

In contrast to the BJA-B cell line, BL60 cells transfected with the pKEX vectors did not express cytokines or the CAT gene at all. This observation was surprising, since integration of the pKEX vectors in the genome of BJA-B was demonstrated by Southern blot analysis. In addition, expression of transfected genes in BL60 cells using a pKEX vector has already been described.[30,31] Possibly the integration process led to destruction of the vector. This was suggested by the results of Southern blot analysis, where the addition of the length of the integrated vector fragments did not result in the length of the whole plasmid.

Maintenance of expression of the EBNA1/oriP vectors was also analyzed without hygromycin B selection pressure. *In vitro* as well as in nude mouse tumors, cytokine expression could be measured over several weeks but, however, declined continuously. In the 4th week after removing hygromycin B the expression level of IL6 was approximately 50% of the initial level, and after about 2 more weeks approximately 25%. Thus, these vectors cannot be used for gene therapeutic strategies where long-term gene expression is essential, as for instance introduction of a multidrug resistance gene in hematopoietic stem cells or substitution of gene defects as in Gaucher's disease. However, for inducing an antitumor immune response by cytokine transfection of tumor cells they represent a suitable tool.

Recently, a vector has been described which also carries the EBV genomic elements EBNA1 and oriP to allow stable, non-integrative maintenance. In addition, this vector carried EBV elements necessary for packaging, thus representing an infectious particle (mini-EBV).[11] The use of infectious particles includes risks difficult to estimate. Naturally, EBV infects B lymphocytes[32] and epithelial cells.[33] It has also been found in T lymphocytes of patients with chronic EBV infection,[34] in parotid gland ducts,[35] in hairy leukoplakia of the tongue[36] and in smooth muscle tumors of immunosuppressed children.[37] Thus, a wide spectrum of cells might be infected when using such mini-EBVs for gene therapy. In addition, since over 90% of the general population harbor the virus, recombination events might occur generating a recombinant EBV carrying the 'therapeutic' genes. Considering the pleiotropic growth stimulating and activating effects of cytokines the use of such mini-EBVs for cytokine-mediated gene therapy might be dangerous.

In contrast, the vectors presented in this work, permit the introduction of cytokine genes in lymphoma cells as non-integrative, self-replicating, extrachromosomal genetic units. Thus, they avoid the hazards of integration into the host cell genome as well as those of using infectious particles. In addition, they are easy to handle and possess a high cloning capacity. Owing to their structural and functional similiarity to the EBV genome these vectors are expected to be functional also, when they contain multiple inserts. Thus, bi- or polycistronic vectors can be constructed, eg vectors containing the cytokine cDNA together with a suicide gene (HSV*tk*) and/or the cDNA encoding for the second signal molecule B7. To restrict the expression of these vectors after transfection the CMV promotor could be replaced through cell-type-specific promoters. The p1-myc promoter for example would lead to expression exclusively in B cells.[38] Taken together, our experiments suggest that these BC219-derived vectors may represent an effective tool for cytokine gene transfer in human lymphoma cells.

Materials and methods

Basic expression vectors

Vector pKEX-2XR (Figure 1a) was described by Rittner *et al*.[29] The 6.8 kb plasmid carries the human cytomegalovirus immediate–early (HCMV-IE) promoter–enhancer element, the simian virus 40 (SV40)-derived small T-antigen splice and late region polyadenylation signal and the hygromycin B resistance gene. Vector BC219 (Figure 1b) was described by Polack *et al*.[39] Briefly, the 13.9 kb plasmid carries the EBV latent origin of replication (oriP), and EBNA1 leading to episomal persistence of the plasmid in eucaryotic cells. Beside the HCMV-IE promoter–enhancer it carries additional enhancer elements for increase of expression, namely the matrix attachment region (MAR), the intron and the 3' enhancer elements of the human Ig kappa light chain gene locus.[39] BC230a contains the chloramphenicolacetyltransferase (CAT) gene as a *Sfi*I fragment and BC-DZ17 contains the *lacZ* gene, cloned into the *Nae*I site of BC219.

Construction of expression vectors

cDNAs of the human cytokines IL6 and TNFα were purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). The CAT gene was kindly provided by M von Knebel-Döberitz (DKFZ Heidelberg, Germany).

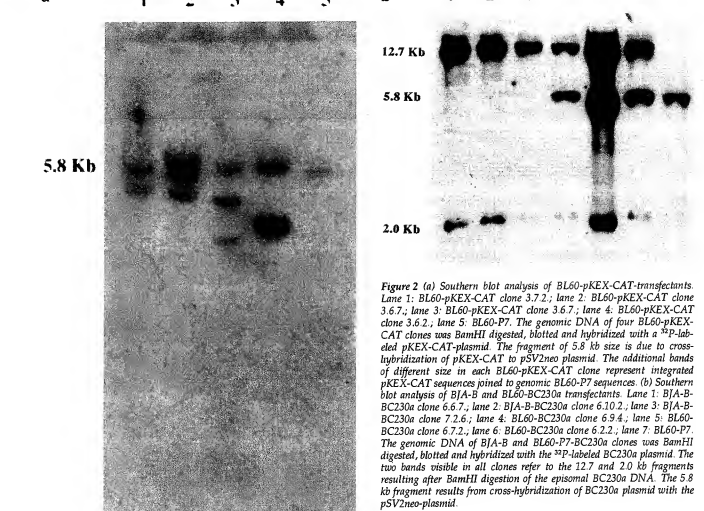

Figure 2 (a) Southern blot analysis of BL60-pKEX-CAT-transfectants. Lane 1: BL60-pKEX-CAT clone 3.7.2.; lane 2: BL60-pKEX-CAT clone 3.6.7.; lane 3: BL60-pKEX-CAT clone 3.6.7.; lane 4: BL60-pKEX-CAT clone 3.6.2., lane 5: BL60-P7. The genomic DNA of four BL60-pKEX-CAT clones was BamHI digested, blotted and hybridized with a ^{32}P-labeled pKEX-CAT-plasmid. The fragment of 5.8 kb size is due to cross-hybridization of pKEX-CAT to pSV2neo plasmid. The additional bands of different size in each BL60-pKEX-CAT clone represent integrated pKEX-CAT sequences joined to genomic BL60-P7 sequences. (b) Southern blot analysis of BJA-B and BL60-BC230a transfectants. Lane 1: BJA-B-BC230a clone 6.6.7., lane 2: BJA-B-BC230a clone 6.10.2., lane 3: BJA-B-BC230a clone 7.2.6.; lane 4: BL60-BC230a clone 6.9.4.; lane 5: BL60-BC230a clone 6.7.2., lane 6: BL60-BC230a clone 6.2.2.; lane 7: BL60-P7. The genomic DNA of BJA-B and BL60-P7-BC230a clones was BamHI digested, blotted and hybridized with the ^{32}P-labeled BC230a plasmid. The two bands visible in all clones refer to the 12.7 and 2.0 kb fragments resulting after BamHI digestion of the episomal BC230a DNA. The 5.8 kb fragment results from cross-hybridization of BC230a plasmid with the pSV2neo-plasmid.

Standard cloning techniques were used as described.[40] For construction of pKEX-CAT the CAT coding sequence was isolated as a *Hin*cII fragment and cloned into the *Sma*I site of pKEX-2XR. For construction of pKEX-IL6 the IL6-cDNA was isolated as an *Eco*RI–*Hin*dIII fragment from the vector pGEMII-2.[41] After blunt-end formation with the Klenow fragment of DNA polymerase I the fragment was inserted into the *Sma*I site of pKEX-2XR. For construction of pKEX-TNFα the TNFα cDNA was isolated as a *Pst*I fragment of the vector pE4,[42] cloned into the Bluescript vector pBS M13 KS (Stratagene, La Jolla, CA, USA), cut out as a *Xho*I–*Xba*I fragment and ligated to the *Xho*I–*Xba*I sites of pKEX-2XR. For cloning cytokine cDNAs into BC219, they were first cloned into pBS*, a variant of pBS M13 KS with a different polylinker site, namely *Sac*I–*Sfi*I–*Pst*I–*Eco*RI–*Xba*I–*Hin*dIII–*Xho*I–*Bam*HI–*Sfi*I–*Kpn*I. It was constructed to provide cytokine cDNAs with a *Sfi*I site before cloning into BC219. BC-IL6 was constructed from the *Eco*RI–*Hin*dIII–IL6 cDNA fragment, BC-GM-CSF from the *Eco*RI–cDNA[43] and BC-TNFα from the *Pst*I–TNFα cDNA.[42]

Plasmid preparation

For transfection, plasmids were amplified in *E. coli* strain Sure cells (Stratagene), isolated using the Ish Horowitz method[40] and purified by cesium chloride gradient centrifugation. The DNA was dialysed against 10 mM Tris, 1 mM EDTA, pH 8 (TE), ethanol-precipitated and resuspended in sterile water.

Cell lines

BL60-P7 is a neomycin-resistant and hypoxanthine-guanine-phosphoribosyltransferase (HGPRT)-deficient subline of the Burkitt's lymphoma cell line BL60.[13] BJA-B is an EBV-negative B cell lymphoma cell line.[44] Cells were cultured in RPMI-1640 medium (GIBCO Laboratories, Grand Island, NY, USA) supplemented with 10% heat-

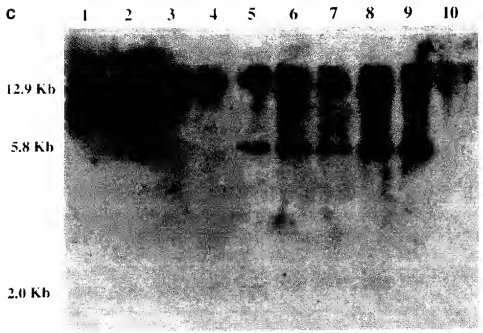

Figure 2 (c) Southern blot analysis of BJA-B and BL60-BC-TNFα transfectants. Lane 1: BJA-B-BC-TNFα clone 6.7.8.; lane 2: BJA-B-BC-TNFα clone 7.1.2.; lane 3: BJA-B-BC-TNFα clone 6.2.6.; lane 4: BJA-B-BC-TNFα clone 7.2.1.; lane 5: BL60-BC-TNFα clone 6.9.2., lane 6: BL60-BC-TNFα clone 7.11.3.; lane 7: BL60-BC-TNFα clone 6.2.7.; lane 8: BL60-BC-TNFα clone 6.7.5.; lane 9: BL60-P7, lane 10: BJA-B. The genomic DNA of BJA-B and BL60-P7-BC-TNFα clones was BamHI digested, blotted and hybridized with the ^{32}P-labeled BC219 plasmid. The two fragments detected of 12.9 and 2.0 kb size represent the BamHI digested BC-TNFα plasmid. The 5.8 kb fragment in the BL60-BC-TNFα clones is due to cross-hybridization of the BC219 plasmid with pSV2neo-plasmid.

Table 3 Secretion of cytokines and expression of CAT protein in BJA-B and BL60 P7 transfectants

Cytokine/marker gene	*Target cell*	*Vector*	*Numbers of clones*	*Gene expression*
CAT	BJA-B	mock	1	negative
		pKEX-CAT	10	90–2100 pg/ml
		BC230a	10	3800–5200 pg/ml
TNFα	BJA-B	mock	1	negative
		pKEX-TNF	9	40–2050 pg/ml
		BC219-TNF	10	500–2100 pg/ml
IL6	BJA-B	mock	1	negative
		pKEX-IL6	10	8–700 pg/ml
		BC219-IL6	10	900–1250 pg/ml
GM-CSF	BJA-B	mock	1	negative
		BC219-GM-CSF	10	655–650 pg/ml
CAT	BL60	mock	1	negative
		pKEX-CAT	5	negative
		BC230a	10	3800–5100 pg/ml
TNFα	BL60	mock	1	negative
		pKEX-TNF	9	negative
		BC219-TNF	10	1350–1900 pg/ml
IL6	BL60	mock	1	negative
		pKEX-IL6	5	negative
		BC219-IL6	10	900–1210 pg/ml
GM-CSF	BL60	mock	1	negative
		BC219-GM-CSF	10	650–655 pg/ml

The values given represent cytokine concentrations in the supernatant of the most productive and the less productive clone. Each value is a mean of two independent measured values. Measurements were performed using ELISA (Quantikine, Biermann, Germany).

Table 4 *In vitro* expression of BL60-BC-IL6 cells with and without hygromycin B selection

	W_0	W_1	W_2	W_3	W_4	W_5	W_6	W_7	W_8	W_9
IL6 + Hygro	456.5	456.5	399.9	454.3	470.2	448.8	459.6	461.3	469.7	440.0
IL6 – Hygro		444.3	358.6	367.7	222.0	52.6	71.5	69.8	17.6	12.5

W_0: IL6 concentration at the beginning of the experiment; W_1: IL6 concentration after 1 week; W_9: IL6 concentration after 9 weeks.

Table 5 IL6 expression of BL60-BC-IL6 cells after inoculation *in vivo*

Tumor size (mm)	*t1: IL6 conc. before s.c. inoculation (pg/ml)*	*t2: IL6 conc. after 2 weeks (pg/ml)*	*t3: IL6 conc. after 4 weeks (pg/ml)*	*t4: IL6 conc. after 6 weeks (pg/ml)*
0 × 0	456.5			
10 × 10 right		212.6		
0 × 0 left		0		
20 × 20 right			66.7	
20 × 20 left			133.0	
25 × 12 right				118.9
30 × 30 left				69.8

inactivated fetal calf serum (FCS), 2 mM glutamine, 100 IU penicilline and 100 μg streptomycin/ml.

Transfection

BL60-P7 and BJA-B cells were transfected using the electroporation method.[45] Briefly, 2×10^7 cells and 20 μg DNA in a total volume of 200 μl PBS were placed in an electroporation chamber with an electrode distance of 4 mm. A high voltage of 200 V (BJA-B), respectively 220 V (BL60-P7), was applied with an electropulsing device, capacitance 1050 microfarads (Easyject, Eurogentec, Belgium). After 5 min on ice, 15 ml of growth medium was added, and cells were incubated for 24 h. Then, cells were plated on 96-well microtiter plates at a concentration of 2×10^5 cells/μl for selection of hygromycin B-resistant clones (400 μg hygromycin/ml medium). Clones of transfected cells were identified 21 days after transfection and were transferred into 24-well microtiter plates.

Enzyme-linked immunosorbent assay (ELISA)

Concentrations of human cytokines IL6, TNFα and GM-CSF in cell culture supernatants were determined by a commercially available ELISA (Quantikine), distributed by Biermann, Bad Nauheim, Germany. Cells were plated 24 h before measurement in fresh medium at a concentration of 1×10^6 cells/μl without hygromycin B. ELISA was performed according to the manufacturer's instructions. The sensitivity thresholds were: 0.35 pg/ml (IL6), 0.17 pg/ml (TNFα) and 1.5 pg/ml (GM-CSF). CAT protein in cell lysates was measured with a CAT-ELISA (Boehringer-Mannheim, Mannheim, Germany) by using 50 μg of total protein extract.

Southern blotting

Extraction of total cellular DNA and restriction endonuclease digestion were performed using standard protocols.[40] After restriction enzyme digestion 10 μg of cleaved

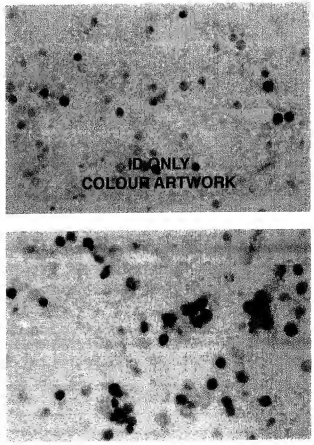

Figure 3 β-Gal assay of BJAB-BC-DZ17 and BL60-BC-DZ17 transfectants. (a) BL60-BC-DZ17 clone 1.3.5.: 50% of the transfected cells show lacZ expression. (b) BJA-B-BC-DZ17 clone 4.3.5.: 80% of the transfected cells show lacZ expression.

cellular DNA were separated by agarose gel electrophoresis and transferred on to a nylon filter (Gene Screen Plus; NEN, Boston, MA, USA). Hybridization was performed in 50% formamide, 2 × SSC at 42°C with ^{32}P-labeled DNA probes.[46] The following probes were used: the pKEX-CAT (Figure 2a), the BC230a plasmid (Figure 2b) and the BC219 plasmid (Figure 2c).

Tumorigenicity assays

Untreated, 4-week-old female nu/nu mice (Swiss background, purchased from Charles River, Wilmington, MA, USA) were inoculated with exponentially growing cells (with a viability of at least 90%). In 0.1 ml RPMI 1640 1×10^7 cells were suspended and subsequently inoculated under the skin of both flanks of untreated, 4-week-old female nu/nu mice . The animals were examined twice weekly and the maximal and minimal diameter of each tumor was measured.

β-Galactosidase (β-gal) assay

For the β-gal assay exponentially growing cells were centrifuged, washed once with PBS, resuspended to a concentration of 2×10^5 cells/μl and transferred on to adhesion slides (Biorad, Munich, Germany). After 10 min, cells were fixed in formaldehyde (3.7%)-PBS, and incubated with an X-gal solution (0.1 M sodium phosphate buffer, pH 7.6, containing 2.5% (v/v) of 40 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (Stratagene) solution in DMSO, 5 mM of both potassium ferricyanide and potassium ferrocyanide, 2 mM $MgCl_2$ at 37° C for 2–24 h. The β-gal reaction was stopped by washing with 0.1 M phosphate buffer.

Acknowledgements

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United States Patent [19]

Levy et al.

[11] Patent Number: 6,099,846

[45] Date of Patent: *Aug. 8, 2000

[54] **ENHANCEMENT OF B CELL LYMPHOMA AND TUMOR RESISTANCE USING IDIOTYPE/CYTOKINE CONJUGATES**

[75] Inventors: **Ronald Levy**, Stanford, Calif.; **Mi-Hua Tao**, Taipei, Taiwan

[73] Assignee: **The Board of Trustees of the Leland Stanford Junior University**, Palo Alto, Calif.

[*] Notice: This patent issued on a continued prosecution application filed under 37 CFR 1.53(d), and is subject to the twenty year patent term provisions of 35 U.S.C. 154(a)(2).

[21] Appl. No.: **08/416,787**

[22] PCT Filed: **Oct. 14, 1993**

[86] PCT No.: **PCT/US93/09895**

§ 371 Date: **Apr. 14, 1995**

§ 102(e) Date: **Apr. 14, 1995**

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PCT Pub. Date: **Apr. 28, 1994**

Related U.S. Application Data

[63] Continuation-in-part of application No. 07/961,788, Oct. 14, 1992, abandoned.

[51] **Int. Cl.**[7] **A61K 39/385**

[52] **U.S. Cl.** **424/195.11**; 424/85.1; 424/85.2; 424/180.1; 530/351; 530/387.3

[58] **Field of Search** 424/85.8, 85.1, 424/85.2, 131.1, 152.1, 178.1, 179.1, 180.1, 193.1, 194.1, 195.11; 435/69.1, 91, 252.3, 320; 530/387.3, 350, 351, 387.1, 387.2, 391.1, 391.5, 806, 808; 536/125.35

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[57] **ABSTRACT**

B cell lymphoma tumor-associated antigen or a fragment thereof containing an epitope are linked to an immune-enhancing cytokine, such as GM-CSF, IL-2, or IL-4 to form an immuno-complex. This immuno-complex elicits immune responses which are protective with respect to tumor proliferation. The linkers may be simple chemical bifunctional moieties introduced through chemical synthetic techniques or peptides introduce through recombinant methodologies. Antibodies immunoreactive with these immunocomplexes are also useful as passive vaccines and as analytical tools.

7 Claims, 10 Drawing Sheets

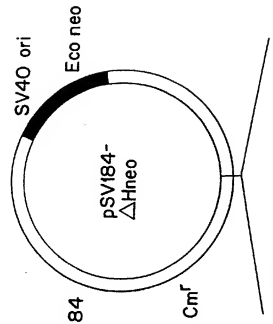
SV40 ori
Eco neo
pSV184-ΔHneo
pACYC184 ori
Cmr
HUMAN Cκ
MURINE Vκ38C13

FIG.1B

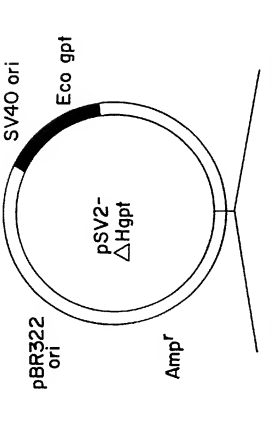
SV40 ori
Eco gpt
pSV2-ΔHgpt
pBR322 ori
Ampr
MURINE VH 38C13
HUMAN IgG Cγ1 H Cγ2 Cγ3
MURINE GM-CSF

FIG.1A

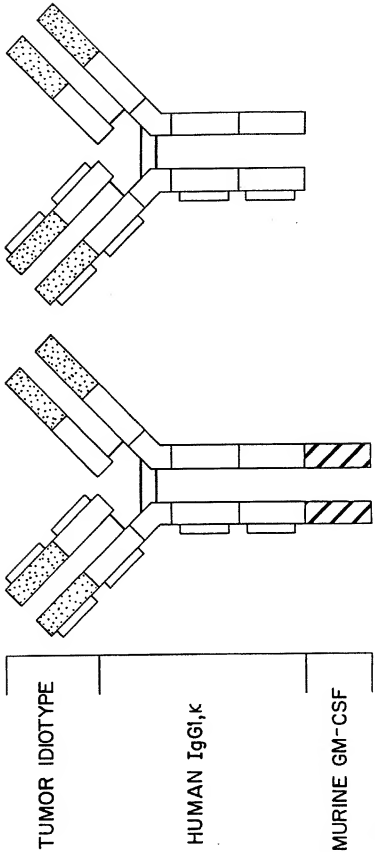

FIG.2

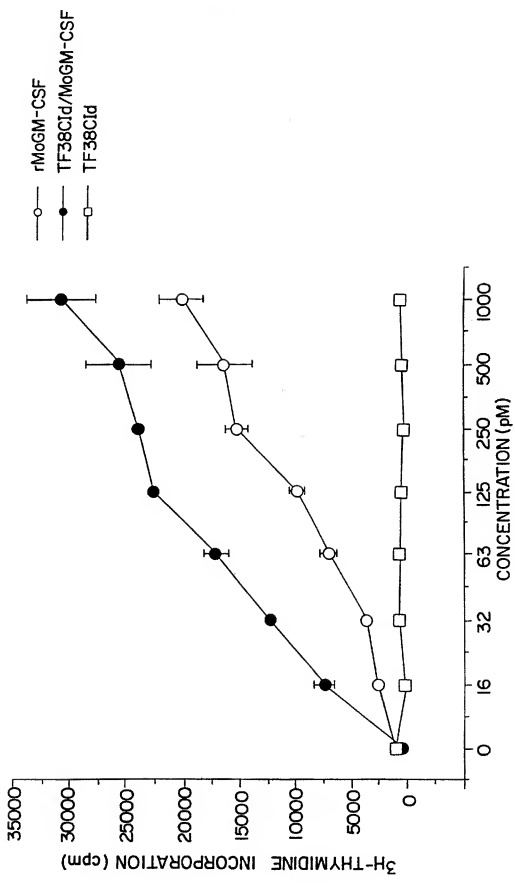
rMoGM-CSF
TF38Cld/MoGM-CSF
TF38Cld
3H-THYMIDINE INCORPORATION (cpm)
35000
30000
25000
20000
15000
10000
5000
0
0
16
32
63
125
250
500
1000
CONCENTRATION (pM)

FIG. 3

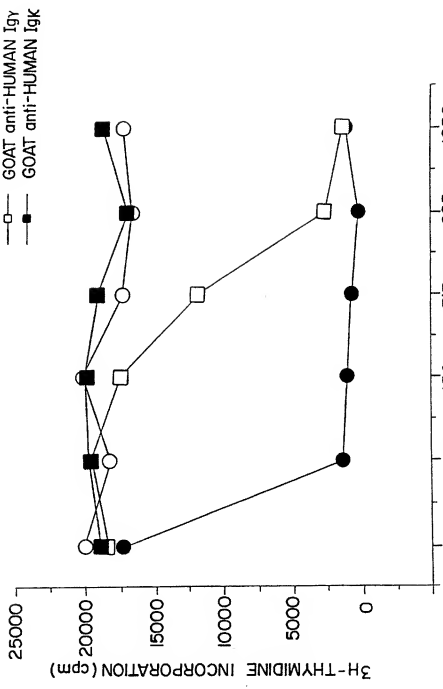
NORMAL RAT IgG
RAT anti-GM-CSF
GOAT anti-HUMAN Igγ
GOAT anti-HUMAN Igκ
3H-THYMIDINE INCORPORATION (cpm)
25000
20000
15000
10000
5000
0
39
78
156
313
625
1250
ANTIBODY CONCENTRATION (ng/ml)

FIG.4

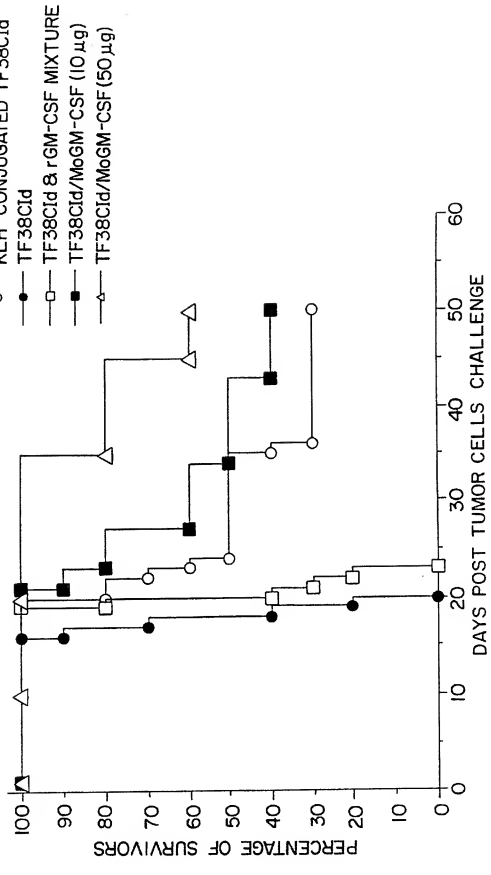
KLH CONJUGATED TF38CId
TF38CId
TF38CId & rGM-CSF MIXTURE
TF38CId/MoGM-CSF (10 μg)
TF38CId/MoGM-CSF (50 μg)
PERCENTAGE OF SURVIVORS
0
10
20
30
40
50
60
70
80
90
100
0
10
20
30
40
50
60
DAYS POST TUMOR CELLS CHALLENGE

FIG.5

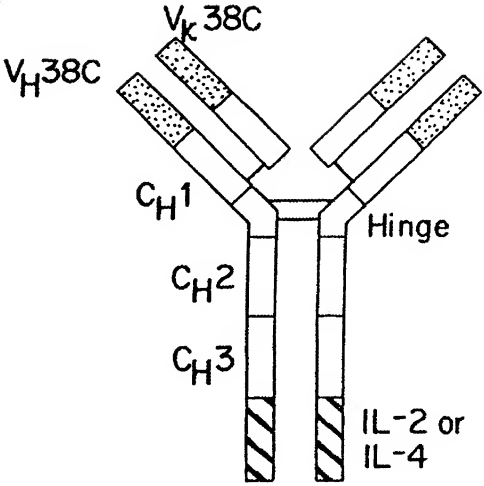

FIG.6A

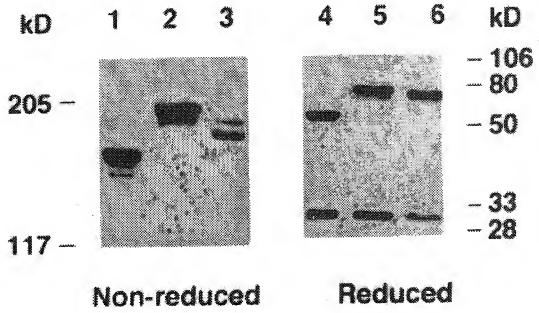
kD
1
2
3
4
5
6
kD
205
117
106
80
50
33
28
Non-reduced
Reduced

FIG.6B

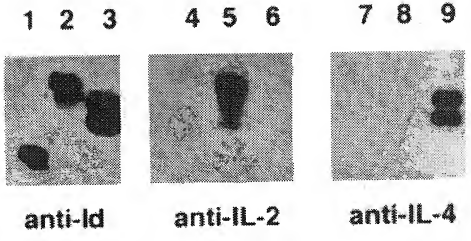
1 2 3
4 5 6
7 8 9
anti-Id
anti-IL-2
anti-IL-4

FIG.6C

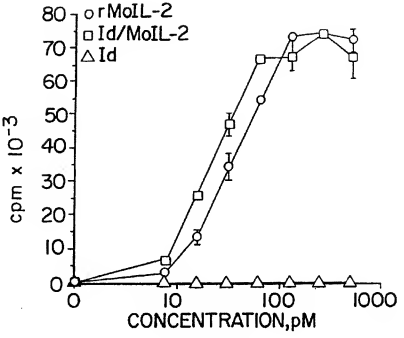
o rMoIL-2
□ Id/MoIL-2
△ Id
cpm x 10⁻³
80
70
60
50
40
30
20
10
0
0
10
100
1000
CONCENTRATION,pM

FIG.7A

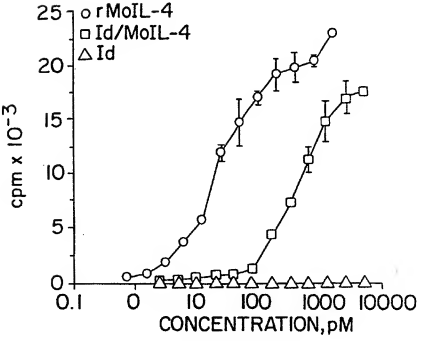
o rMoIL-4
□ Id/MoIL-4
△ Id
cpm x 10⁻³
25
20
15
10
5
0
0.1
0
10
100
1000
10000
CONCENTRATION,pM

FIG.7B

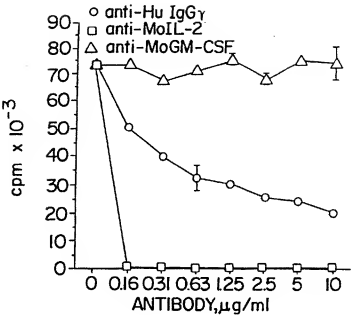
o anti-Hu IgGγ
□ anti-MoIL-2
△ anti-MoGM-CSF
cpm x 10⁻³
90
80
70
60
50
40
30
20
10
0
0 0.16 0.31 0.63 1.25 2.5 5 10
ANTIBODY, μg/ml

FIG.7C

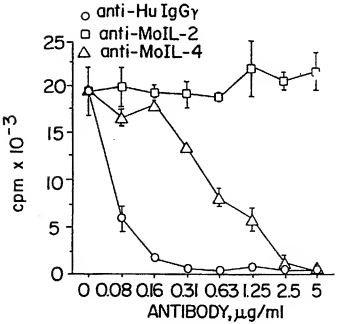
o anti-Hu IgGγ
□ anti-MoIL-2
△ anti-MoIL-4
cpm x 10⁻³
25
20
15
10
5
0
0 0.08 0.16 0.31 0.63 1.25 2.5 5
ANTIBODY, μg/ml

FIG.7D

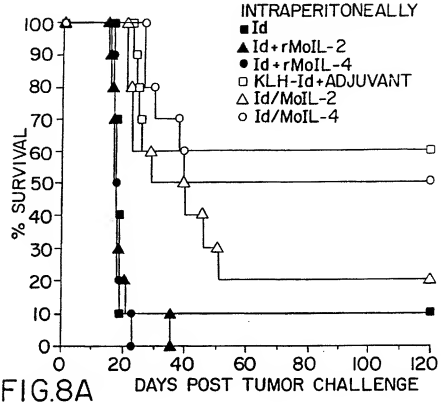
INTRAPERITONEALLY
Id
Id+rMoIL-2
Id+rMoIL-4
KLH-Id+ADJUVANT
Id/MoIL-2
Id/MoIL-4
% SURVIVAL
DAYS POST TUMOR CHALLENGE

FIG.8A

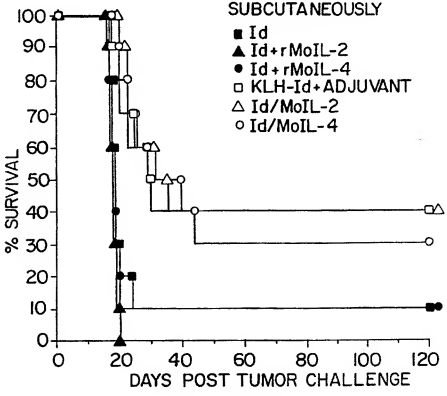
SUBCUTANEOUSLY
Id
Id+rMoIL-2
Id+rMoIL-4
KLH-Id+ADJUVANT
Id/MoIL-2
Id/MoIL-4
% SURVIVAL
DAYS POST TUMOR CHALLENGE

FIG.8B

ENHANCEMENT OF B CELL LYMPHOMA AND TUMOR RESISTANCE USING IDIOTYPE/CYTOKINE CONJUGATES

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a 35 U.S.C. §371 filing of International Patent Application No. PCT/US93/09895, which is a continuation-in-part of U.S. Ser. No. 07/961,788, filed Oct. 14, 1992, now abandoned.

TECHNICAL FIELD

The invention relates to the field of modulating B cell lymphoma tumor growth using immunological methods. More specifically, the invention concerns use of immunological constructs to confer resistance to B cell lymphoma tumors, and to enhance immune response.

BACKGROUND ART

Malignant tumors often express characteristic antigens or "markers" which offer a mechanism for tumor prevention, resistance or treatment. The antigens which are characteristic of the tumor may be purified and formulated into vaccines. This may stimulate an antibody response and a cellular immune response which are helpful in controlling tumor growth. At a minimum, the antibodies raised by these antigens can be used as detection tools to monitor the level of tumor marker in the host to track the course of the disease or the effectiveness of treatment.

It is well known that the immunogenicity of antigens can be enhanced by coupling these hapten-bearing moieties to carriers. A variety of carriers are routinely used, such as keyhole limpet hemocyanin and various serum albumins. It is also understood that certain cytokines, such as GM-CSF, have the capacity to enhance primary antibody responses to antigens (Morrissey, P. J., et al, *J Immunol* (1987) 139: 1113–1119).

The immune response-enhancing ability of a cytokine, when coupled to a viral antigen, has been shown by Hinuma, S., et al, *FEBS* (1991) 288: 138–142. In this work, interleukin-2 was coupled to a herpes simplex virus type I glycoprotein by generating a fusion protein consisting of the glycoprotein D and human IL-2. The conjugate was shown to induce high antibody responses and cell-mediated immunity to HSV-I in mice.

It has now been found that B cell lymphoma tumor-associated antigens can be coupled to immune response-enhancing cytokines, such as GM-CSF, IL-2 and IL-4, to produce an immune response to the tumor antigen and enhance the ability of the host to resist tumor growth associated with the antigen.

DISCLOSURE OF THE INVENTION

The invention provides compositions and methods for the modulation of B cell lymphoma tumor growth where the tumor is characterized by an associated antigen carrying an epitope which is characteristic of the tumor. The immunogenicity of preparations of the antigen used to raise antibodies and stimulate an immune response can be enhanced by coupling the antigen to an appropriate cytokine. The conjugate is superior in effect to the antigen coupled to conventional immunogenic carriers.

In one aspect, the invention is directed to an immunocomplex, which complex comprises a B cell lymphoma tumor-associated antigen covalently coupled to an immune-enhancing cytokine. The complex may be obtained by generating the antigen and the cytokine as a fusion protein using recombinant techniques; thus, in another aspect, the invention is directed to recombinant materials and methods for production of such fusion proteins. In still other aspects, the invention is directed to pharmaceutical compositions and vaccines containing the immunogenic complexes of the invention and to methods of conferring antitumor immunity using these complexes. In still other aspects, the invention is directed to antibodies generated by immunization with the complexes of the invention and to antibodies immunospecific to the conjugates. The invention is also directed to methods to confer immunity by administering polyclonal or monoclonal preparations of antibodies generated by the conjugates.

It has also been found that the immune-enhancing cytokine activity is improved by extending or coupling the cytokine to an additional moiety. Accordingly, in another aspect, the invention is directed to enhancing the activity of a cytokine by coupling to an additional moiety.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1A** shows diagrammatically the construction of expression vectors for a B cell lymphoma tumor antigen Ig heavy chain coupled to murine GM-CSF;

FIG. **1B** shows diagrammatically the construction of an expression vector for the light chain of this tumor-associated antigen.

FIG. **2** shows diagrammatically the products of transformed mouse myeloma cells transfected with the vectors illustrated in FIGS. **1A** and **1B**.

FIG. **3** is a graph showing the ability of GM-CSF-coupled antigen to support the proliferation of a GM-CSF-dependent cell line.

FIG. **4** is a graph which shows the ability of antibodies produced against GM-CSF to inhibit the proliferative activity of the immunocomplex.

FIG. **5** is a graph showing the ability of mice immunized with the immunocomplex of the invention to resist tumor challenge.

FIG. **6A** shows the dimeric Id/interleukin fusion proteins.

FIG. **6B** is a photocopy of a photograph of the chimeric tumor idiotypic/interleukin proteins analyzed by gel electrophoresis.

FIG. **6C** is a photocopy of a photograph of the immunoblotting analysis of the chimeric idiotypic/interleukin proteins.

FIG. **7A** shows the results of an analysis of the purified chimeric idiotypic/interleukin proteins for their ability to support the proliferation of a murine IL-2/IL-4-responsive T cell line, HT-2.

FIG. **7B** is a comparison of the activity of recombinant IL-4, the idiotypic/IL-4 chimeric protein and the idiotypic antigen in the proliferation assay described above.

FIGS. **7C** and **7D** show that the activity of Id/MoIL-2 and Id/MoIL-4 was inhibited by anti-IL-2 and anti-IL-4 antibodies, respectively, but not by a control antibody.

FIG. **8A** illustrates that mice vaccinated intraperitoneally with Id/MoIL-2 or Id/MoIL-4 had a significantly prolonged survival time compared with mice receiving Id alone.

FIG. **8B** illustrates that mice immunized with Id/MoIL-2 and Id/MoIL-4 also induced protective immunity and resulted in 40% and 30% long-term survivors, respectively, while vaccination with a mixture of Id and IL-2 or IL-4 yielded no protection.

MODES OF CARRYING OUT THE INVENTION

The invention provides B cell lymphoma antitumor vaccines of enhanced immunogenicity wherein the active ingredient is an immunocomplex containing an immune-enhancing cytokine covalently bound to a B cell lymphoma tumor-associated antigen or a fragment thereof containing at least one epitope characteristic of the tumor.

By "tumor-associated antigen" is meant a proteinaceous molecule containing at least one epitope wherein the epitope characterizes the tumor and is unique to the tumor as opposed to other tissues. The nature of the tumor-associated antigen will, of course, vary with the nature of the tumor. For B-cell lymphomas, the tumor-associated antigen is most frequently an immunoglobulin. The use of such tumor-associated antigens to produce monoclonal anti-idiotype antibodies has been described in U.S. Pat. No. 4,661,586 and U.S. Pat. No. 4,816,249. An assay for the monoclonal antibodies against tumor surface immunoglobulins is described and claimed in U.S. Pat. No. 4,513,088. The disclosure of these patents is incorporated herein by reference.

By "immune-enhancing cytokine" is meant a cytokine that is capable of enhancing the immune response when the cytokine is generated in situ or is administered to a mammalian host. Such cytokines are well known in the art and have become numerous. They include GM-CSF, IL-2, IL-3, and IL-4.

The immune-enhancing activity of cytokines can also be improved by their extension with additional moieties. As shown in Example 2, a cytokine coupled to an additional molecular structure is more effective in stimulating proliferation of NFS-60 cells than the cytokine alone. Such additional molecular structure is variable, and may comprise additional copies of the cytokine itself.

The tumor-associated antigen or the relevant epitope-bearing portion thereof may be coupled to the immune-enhancing cytokine using conventional coupling techniques such as coupling with dehydrating agents such as ECDI, dicyclohexylcarbodiimide (DCCI), and the like. In addition, commercially available or other synthetic linkers may be used in standard chemical coupling techniques. Such linkers, which are capable of coupling through sulfhydryl groups, amino groups, or carboxyl groups, are available from Pierce Chemical Co. (Rockford, Ill.). If the tumor-associated antigen or cytokine contains glycosylation, coupling can also be effected through the carbohydrate moieties using, for example, reductive amination. A wide variety of standard coupling techniques for covalent binding of the B cell lymphoma tumor-associated antigen with the cytokine is known in the art.

The B cell lymphoma tumor-associated antigen may be obtained by purification from the tumor or may be synthesized using recombinant techniques. In the case of the B-lymphomas, the immunoglobulin antigen may be affinity purified using, for example, protein A or other suitable means. A wide variety of cytokines is available in the art, and the genes for many of the cytokines have been cloned so that recombinant production of the cytokine portion of the complex may also be effected.

Alternatively, the immunocomplex can be constructed recombinantly as a fusion protein using the genes encoding the B cell lymphoma tumor-associated antigen and the immune-enhancing cytokine. Such fusions can be constructed by ligating DNAs encoding each protein in reading frame and coupling these to standard expression systems, as is by now generally known in the art. In general, it is preferred to produce the fusion proteins in mammalian host cells, although procaryotic or yeast expression is also feasible. The choice of control sequences that will be operably linked to the DNA encoding the fusion protein will depend on host choice, and the wide availability of control sequences for selected hosts is by now well known.

The immunocomplexes of the invention can then be used to engender an immune response in the host to be protected against the tumor. Generally, the immunocomplex is administered by injection—intravenous, intramuscular, intraperitoneal and the like—along with a suitable vehicle and excipient for injection. Suitable formulations for injection, including appropriate adjuvants, can be found, for example, in *Remington's Pharmaceutical Sciences*, Mack Publishing Company, Easton, Pa., latest edition. Protocols for administration can be formulated using routine optimization techniques, whereby repeated administration and concomitant antibody titering of the serum is performed until suitable titers are obtained. The polyclonal antisera produced by administration of the immunocomplexes of the invention can also be used for passive immunization of hosts against tumor proliferation. Preferably, however, the antibody-producing cells of the immunized host are immortalized, for example, using the standard fusion techniques of Kohler and Milstein, and the resulting immortalized cells are screened for production of antibodies which are immunoreactive with the B cell lymphoma tumor-associated antigen.

By "immunoreactive with the B cell lymphoma tumor-associated antigen" is meant that the antibodies have sufficiently high affinity for such antigens to detect them in standard assay systems. Such polyclonal or monoclonal antibodies are also useful in passive immunization.

Antibodies will also be produced which are immunospecific for the immunocomplexes. By "immunospecific for the immunocomplex" is meant that the antibodies recognize the immunocomplex as distinct from the components thereof. These immunospecific antibodies are useful in monitoring the levels of immunocomplex circulating in the plasma as well as in monitoring the production of the immunocomplex by expression of the appropriate recombinant systems. These antibodies may also be conveniently used for purification of the immunocomplex from the production vessels.

The following examples are intended to illustrate but not to limit the invention.

EXAMPLE 1

Construction of Idiotype/GM-CSF Fusion Protein

A carcinogen-induced murine B-cell tumor, 38C13, was used as the source of tumor-derived idiotypic protein. Immunoglobulins produced by this tumor, 38C1d, have been used in immunization protocols and shown to be effective in protecting animals from subsequent lethal tumor challenge. In these immunizations, the antigen was coupled to standard immunogenic carriers such as KLH.

The V_H and V_L genes of the 38C13 tumor cells were cloned using the polymerase chain reaction (PCR) and ligated to human Igγ and Igκ constant region genes in the heavy- and light-chain expression vectors pSV2-ΔHGPT and pSV184-ΔHneo, respectively. The murine GM-CSF gene was cloned by PCR and ligated to the Cγ3 exon of the heavy-chain gene. The construction of these vectors is shown diagrammatically in FIGS. 1A and 1B, respectively. FIG. 1A shows diagrammatically the coupling of the V_H region of 38C13 and the Cγ1, Hγ, Cγ2, and Cγ3 regions of human IgG. The heavy-chain DNA is constructed including

or not including the coding sequence for murine GM-CSF. The constructs are then inserted into the host vector as shown.

FIG. 1B shows the coupling of the murine V_κ 38C13 with the human C_κ constant region. The hybrid gene is ligated into pSV184-ΔHneo under control of the host vector promoter.

Both plasmids are then transfected by electroporation into the Ig-deficient murine plasma-cytoma cell line AG8.653 for expression. The culture supernatants were screened for production of the chimeric proteins using a standard ELISA assay, and the chimeric proteins were purified by protein-A chromatography.

FIG. 2 shows diagrammatically the proteins produced. TF38CId is the tumor-associated antigen comprising the tumor idiotype coupled with constant regions derived from human immunoglobulins. TF38CId/MoGM-CSF is identical, except for the fusion to murine GM-CSF. The chimeric proteins secreted by the murine myeloma cells were analyzed by reducing and nonreducing SDS-PAGE. Analysis of the results showed that TF38CId/MoGM-CSF was expressed as a molecule with a molecular weight of 210 kd.

EXAMPLE 2

Ability of the Fusion Protein to Retain Immune Enhancement

Proliferation assays were conducted by plating GM-CSF-dependent NFS-60 cells with various concentrations of test compound. The cells were incubated for 18 hours and the proliferation determined by standard labeled thymidine-incorporation assay.

FIG. 3 shows the results of this assay. The graph in FIG. 3 plots thymidine incorporation in counts per minute (Y-axis) against the concentration in pM (X-axis). Treatment with TF38CId alone (□) provides no stimulation of proliferation; treatment with recombinant murine GM-CSF alone (○) shows intermediate ability to proliferate; treatment with the fusion protein of the invention, TF38-CId/MoGM-CSF (●) shows greatly enhanced ability to stimulate proliferation in a dose-dependent fashion.

The ability of the fusion protein to stimulate cell proliferation could, as expected, be inhibited by antibodies raised against GM-CSF and, to a lesser extent, by antibodies raised against the constant heavy chain to which the GM-CSF is fused. In this assay, NFS-60 cells were incubated with 250 pM of the fusion protein and with various antibodies. The proliferation was determined by thymidine uptake, as above.

FIG. 4 shows the results of these assays where antibody concentration in ng/ml is plotted on the X-axis against thymidine incorporated on the Y-axis. As shown, neither antibodies to human constant κ-chain (■) or to normal rat immunoglobulin (○) were able to inhibit the proliferation of NFS-60 cells stimulated by the fusion protein of the invention. However, proliferation was almost completely inhibited by 78 ng/ml rat anti-GM-CSF (●) and, to a lesser extent, by goat antihuman Ig-γ (□) wherein 625 ng/ml of these antibodies diminished proliferation almost to zero.

EXAMPLE 3

Ability of the Fusion Protein to Immunize Mice

Groups of C3H/HeN mice were immunized twice intraperitoneally with either KLH-conjugated TF38CId, unconjugated TF38CId, TF38CId mixed with recombinant murine GM-CSF, or with the fusion protein of the invention, TF38CId/MoGM-CSF. The immunogens were administered at days −28 and −14 before tumor challenge at day 0. The animals were bled at days −18 and −4 and anti-idiotypic antibodies determined by ELISA against native 38CId (mouse IgM, κ). The antibody titers are shown in Table 1.

TABLE 1

IMMUNOGEN	ANTI-ID (μg/ml ± SD) DAY (−18)	ANTI-ID (μg/ml ± SD) DAY (−4)
KLH-conjugated TF38CId	23 ± 14	296 ± 207
TF38CId	0	0
TF38CId & rMOGM CSF Mixture	0	0
TF38CId/MoGM-CSF	55 ± 39	152 ± 139

As seen in the table, both the KLH-conjugated idiotype and the protein of the invention raised substantial antibody titers; the KLH-conjugated complex was slightly more effective.

However, the fusion protein was more successful in prolonging survival after challenge with tumor cells. At day 0, the mice were challenged with 38C13 tumor cells intraperitoneally, and the survival of the challenged mice was followed. The results are shown in FIG. 5.

Mice immunized with idiotype alone (TF38CId) or with idiotype mixed with recombinant GM-CSF (● and □, respectively) survived only 20–25 days after challenge. All animals were dead after that time. Animals immunized with KLH-conjugated idiotype (○) had a 50% survival rate at day 25 and 30% of the animals were still alive after 50 days. Mice immunized with 10 μg of fusion protein (●) had a 50% survival rate after 34 days and 40% were still alive at day 50. Mice immunized with 50 μg of the fusion protein (Δ) had a 60% survival rate at day 50.

EXAMPLE 4

Construction of Idiotype/IL-2 and IL-4 Fusion Proteins

To make Id/MoIL-2 and Id/MoIL-4 fusion proteins, the genetic fragment encoding mature murine IL-2 or IL-4 was attached to the end of the C_H3 exon of the human Cγ1 gene. The resulting fusion proteins were dimeric with respect to the interleukin molecule as shown in FIG. 6A. All proteins were produced in tissue culture and purified by protein A chromatography. These chimeric tumor idiotypic proteins were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting techniques. Under reducing conditions the protein bands of Id migrate with apparent mw of 30.5- and 58-kD, representing the heavy and light chains, respectively (FIG. 1B). Reduction of Id/MoIL-2 and Id/MoIL-4 also gave a 30.5-kD light chain, but both heavy chain molecules migrated slower than Id heavy chain with apparent mw of 76- and 72-kD, respectively, indicating that they contained the IL-2 or IL-4 tail. As shown in the nonreducing gel (FIG. 6B), the Id protein was secreted as a single band of mw of 160-kD. Both fusion proteins were migrated as doublets in the nonreducing gel with apparent mw of 191- and 198-kD (Id/MoIL-2) and 181- and 191-kD (Id/MoIL-4), indicating that these molecules are present in two different conformations. The immunoblotting analysis of these chimeric idiotypic pro-

teins is shown in FIG. 6C. The results were consistent with the expected constructs of these proteins, in that the anti-Id antibody recognizes all three Id-derived molecules; in contrast, anti-IL-2 and anti-IL-4 antibodies only recognize Id/MoIL-2 and Id/MoIL-4 fusion molecules, respectively.

EXAMPLE 5

Ability of the IL Fusion Proteins to Retain Immune Enhancement

ELISA assay results showed that Id, Id/MoIL-2, and Id/MoIL-4 all reacted with four different monoclonal anti-idiotypic antibodies raised against the native murine 38C13 idiotypic protein. To determine the functional activity of the murine IL-2 or IL-4 portion of the fusion molecules, purified proteins were analyzed for their ability to support the proliferation of a murine IL-2/IL-4-responsive T cell line, HT-2. While Id was completely negative in this assay, Id/MoIL-2 clearly demonstrated the ability to stimulate the growth of HT-2 cells in a dose-dependent manner (FIG. 7A). On a per molecule basis, Id/MoIL-2 was indistinguishable from recombinant murine IL-2 in the ability to induce the proliferation of HT-2 cells. When compared to recombinant IL-4, Id/MoIL-4 fusion protein had about a 25-fold decrease in the activity (FIG. 7B). The reduction in specific activities is not explained by the low pH used for eluting Id fusion proteins from protein A Sepharose, since the activity of the Id/MoIL-4 from the transfected cell culture media and the purified protein is almost identical. Mouse IL-4 contains three sets of disulfide bonds which are important for maintaining its structure and the biological activities. Fusion of IL-4 to the immunoglobulin heavy chain may disrupt the normal disulfide pairings and thus decrease its activity. The fact that two conformational Id/MoIL-4 molecules are present in the purified proteins supports this hypothesis. FIG. 7C and 7D show that the activity of Id/MoIL-2 and Id/MoIL-4 was inhibited by anti-IL-2 and anti-IL-4, respectively, but not by a control antibody, indicating that the cytokine activity is specific. Incubation of the Id/MoIL-2 or Id/MoIL-4 with antibody against human γ-chain also arrested cell growth, indicating that the cytokine activity is in the form of a fusion molecule.

EXAMPLE 6

Ability of the IL Fusion Proteins to Immunize Mice

Anti-idiotypic antibodies were induced by immunization with different idiotypic proteins. Groups of eight- to ten-week-old female C3H/HeN mice were immunized intraperitoneally or subcutaneously with the indicated immunogens in phosphate-buffered saline unless otherwise indicated. Ten mice were included in each group. Two weeks later mice were boosted with the same antigens. KLH-Id was made by coupling KLH to Id using 0.1% glutaraldehyde#. The cytokine molarity of 1.4 μg of recombinant murine IL-2 (rMoIL-2) and 1.9 μg of rMoIL-4 was equivalent to 10 μg of Id/MoIL-2 and Id/MoIL-4, respectively. Serum was sampled by tail bleeding ten days after the last immunization. The titers of antisera against native 38C13 idiotype (IgM, κ) were determined by ELISA using a cocktail of murine antibodies [S3H5 (IgG1), S1C5 (IgG2a), S5A8 (IgG2b)] as a standard. The results are shown in Table 2. The data are expressed as the mean concentration±standard deviation (SD).

TABLE 2

Immunogen	Dose (μg)	Anti-idiotypic antibody (μg/ml ± SD) Intraperit.	Subcutan.
Id	10	0	0
Id + rMoIL-2	10 + 1.4	0	0
Id + rMoIL-4	10 + 1.9	0	0
KLH-Id + sdj.*	10	205.7 ± 39.0	139.8 ± 19.0
Id/MoIL-2	10	18.3 ± 10.4	28.7 ± 16.2
Id/MoIL-4	10	79.0 ± 25.6	45.1 ± 15.9

*KLH-Id was administered with the adjuvant SAF-1 in the first immunization and with incomplete SAF-1 (lacking the MDP component) in the second immunization.

Sera from animals immunized with Id and its cytokine-derived fusion proteins, either intraperitoneally or subcutaneously, were collected 10 days after the second immunization and analyzed in an ELISA for their reactivity with native 38C13 idiotypic protein. Id did not induce detectable anti-idiotypic antibodies (Table 2); thus the carrier or helper effects of human immunoglobulin constant regions were negligible. Conversely, both Id/MoIL-2 and Id/MoIL-4 induced significant amounts of anti-idiotypic antibodies in all immunized animals. Vaccination of Id/MoIL-4 via the intraperitoneal route elicited higher titers of anti-idiotypic antibodies (79.0±25.6 μg/ml) compared to that induced through the subcutaneous route (45.1±15.9 μg/ml), while no significant difference of antibody titers was found in mice immunized with Id/MoIL-2 either intraperitoneally or subcutaneously. Animals immunized with a simple mixture of Id and recombinant murine IL-2 or IL-4 produced no anti-idiotype response, indicating that fusion of the cytokine molecule to tumor idiotype was required for enhanced immunogenicity. The carrier effects of murine IL-2 or murine IL-4 in the fusion protein in the immune responses was ruled out since fusion proteins containing a similar size but biologically inert human GM-CSF or a peptide derived from the pre-S2 region of hepatitis B virus surface antigen # induced no anti-idiotypic antibodies. These results show that the fusion protein requires cytokine activity for its immune-enhancing function. Compared with mice immunized with Id chemically conjugated to the strong carrier protein keyhole limpet hemocyanin (KLH) with adjuvant, titers of anti-idiotypic antibodies induced by Id/MoIL-4 and Id/MoIL-2 were about three-fold and five- to seven-fold lower, respectively. The immune responses are specific because no reactivity occurred against other proteins such as KLH or 4C5 (an irrelevant mouse IgM). Fluorescent-activated cell sorting (FACS) analysis showed that the immune sera from mice vaccinated with Id/MoIL-2 or Id/MoIL-4 specifically recognized 38C13 tumor cells but not an immunoglobulin-negative variant.

Two weeks after the second immunization, mice were challenged with 38C13 tumor cells. FIG. 8A illustrates that mice vaccinated intraperitoneally with Id/MoIL-2 or Id/MoIL-4 had a significantly prolonged survival time compared with mice receiving Id alone ($p<0.01$ and $p<0.01$). 50% of Id/MoIL-4 and 20% of Id/MoIL-2 immunized animals remained tumor free for greater than 120 days, whereas vaccination with Id yielded only 10% long-term survivors. Mice immunized with Id along with recombinant IL-2 or IL-4 did not show any protection ($p>0.7$ and $p>0.2$) and all mice died of tumors before day 36. The positive control group receiving KLH-conjugated Id with adjuvant were well protected and resulted in 60% survivors ($p<0.01$) (FIG. 8A). Similar results were obtained from groups of

mice immunized subcutaneously. As shown in FIG. **8B**, mice immunized with Id/MoIL-2 and Id/MoIL-4 also induced protective immunity and resulted in 40% and 30% long-term survivors, respectively. Again, vaccination with a simple mixture of Id and IL-2 or IL-4 yielded no protection. The long-term survivors (>120 days after tumor challenge) were free of residual or dormant tumor cells by several criteria, including FACS analysis, in vitro culture and in vivo transfer of splenocytes. Tumors that did grow in the immunized animals were examined for surface idiotype expression and were found to be positive.

What is claimed is:

1. An immunocomplex consisting of a B cell lymphoma tumor-associated idiotypic immunoglobulin covalently bound directly or through a linker to an immune-enhancing cytokine, wherein said immunocomplex induces the formation of protective antibody specific to the B cell lymphoma tumor associated idiotypic immunoglobulin.

2. The immunocomplex of claim **1** wherein said B cell lymphoma tumor-associated idiotypic immunoglobulin and said immune-enhancing cytokine are coupled through a nonprotein linker.

3. The immunocomplex of claim **1** wherein said B cell lymphoma tumor-associated idiotypic immunoglobulin and said immune-enhancing cytokine are linked through a protein linker, said immunocomplex being a fusion protein.

4. The immunocomplex of claim **1** wherein the immune-enhancing cytokine is GM-CSF.

5. The immunocomplex of claim **1** wherein the immune-enhancing cytokine is IL-2.

6. The immunocomplex of claim **1** wherein the immune-enhancing cytokine is IL-4.

7. A vaccine for protecting subjects against B cell lymphoma tumor proliferation, which vaccine comprises an effective amount of the immunocomplex of claim **1** in admixture with at least one pharmaceutically acceptable excipient.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 6,099,846
DATED : August 8, 2000
INVENTOR(S) : Ronald Levy and Mi-Hau Tao

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 1,
Line 11, above the "TECHNICAL FIELD" heading, please insert the following heading: -- STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT --; and
Above the "TECHNICAL FIELD" heading, please insert the following paragraph:
-- This invention was made with Government support under contract CA33399 awarded by the National Institutes of Health. The Government has certain rights in this invention. --.

Signed and Sealed this

Twenty-fourth Day of June, 2003

JAMES E. ROGAN
Director of the United States Patent and Trademark Office

US005650150A

United States Patent [19]

Gillies

[11] Patent Number: 5,650,150

[45] Date of Patent: Jul. 22, 1997

[54] **RECOMBINANT ANTIBODY CYTOKINE FUSION PROTEINS**

[76] Inventor: **Stephen D. Gillies**, 245 Leavitt St., Hingham, Mass. 02043

[21] Appl. No.: **281,238**

[22] Filed: **Jul. 27, 1994**

Related U.S. Application Data

[63] Continuation of Ser. No. 788,765, Nov. 7, 1991, abandoned, which is a continuation-in-part of Ser. No. 612,099, Nov. 9, 1990, abandoned.

[51] **Int. Cl.**[6] **A61K 39/395**; A61K 39/40; A61K 45/05; C12P 21/04

[52] **U.S. Cl.** **424/134.1**; 424/133.1; 424/85.1; 435/69.7

[58] **Field of Search** 530/387.1, 387.3, 530/388.3, 387.8; 424/134.1, 133.1, 159.1, 147.1, 155.1; 435/69.7

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Primary Examiner—Lila Feisee
Assistant Examiner—Ray F. Ebert
Attorney, Agent, or Firm—Testa, Hurwitz & Thibeault, LLP

[57] **ABSTRACT**

Immunoconjugates for the selective delivery of a cytokine to a target cell are disclosed. The fusion proteins are comprised of an immunoglobulin heavy chain having a specificity for the target cell, such as a cancer or virus-infected cell, and a cytokine, such as lymphotoxin, tumor necrosis factor alpha, interleukin-2, or granulocyte-macrophage colony stimulating factor, joined via its amino terminal amino acid to the carboxy-terminus of the immunoglobulin. Nucleic acid sequences encoding these fusion proteins and methods of their preparation by genetic engineering techniques are also disclosed.

21 Claims, 9 Drawing Sheets

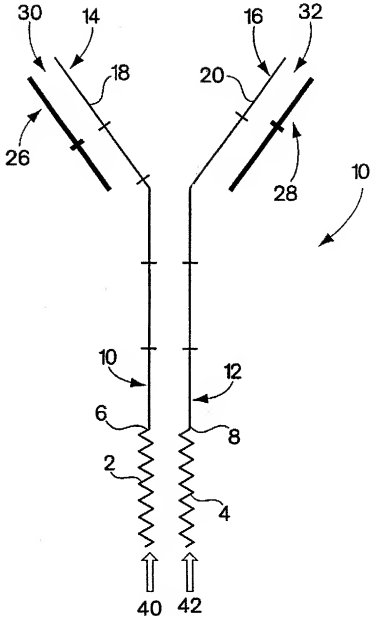


Fig. 1

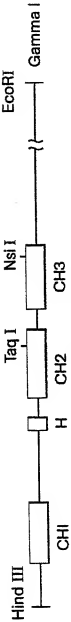


Fig. 2A

Fig. 2B

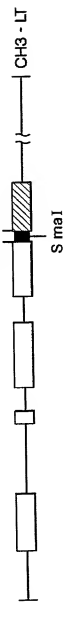


Fig. 2C

Fig. 2D

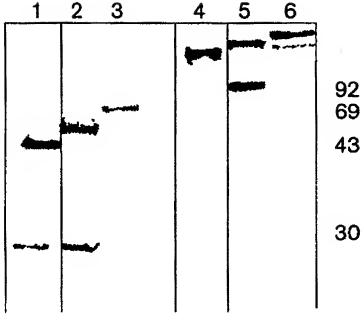


Fig. 3

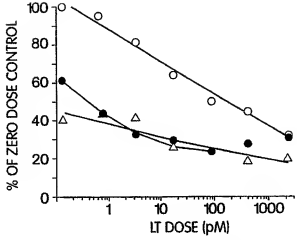


Fig. 4A

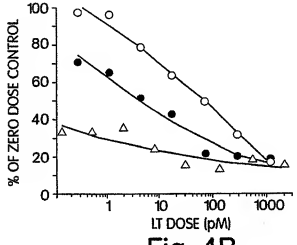


Fig. 4B

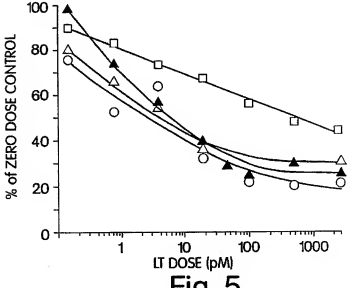
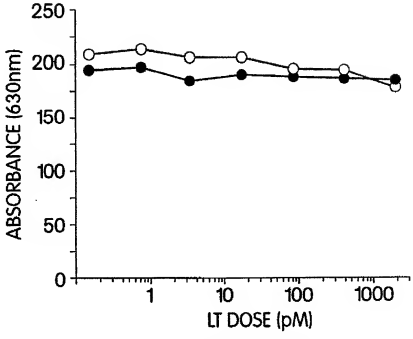


Fig. 5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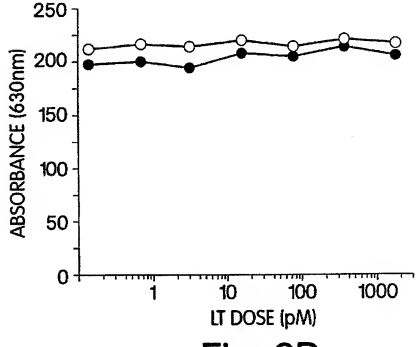
LT DOSE (pM)

Fig. 6A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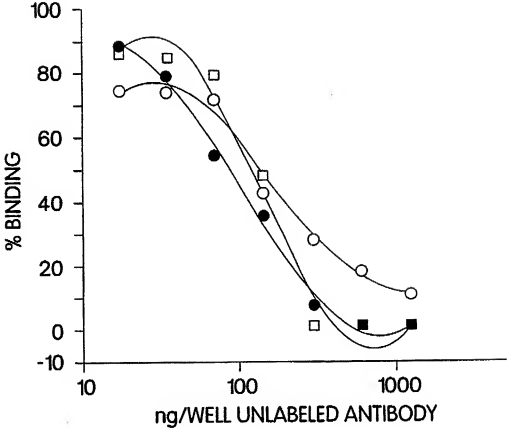
LT DOSE (pM)

Fig. 6B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60
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20
0
-10
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10
100
1000
ng/WELL UNLABELED ANTIBODY

Fig. 7

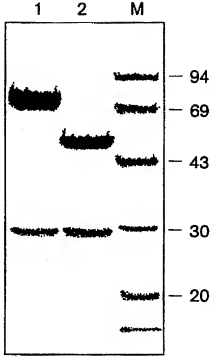


Fig. 8A

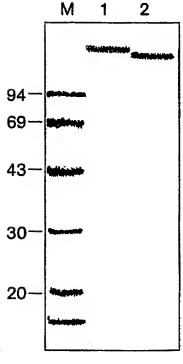
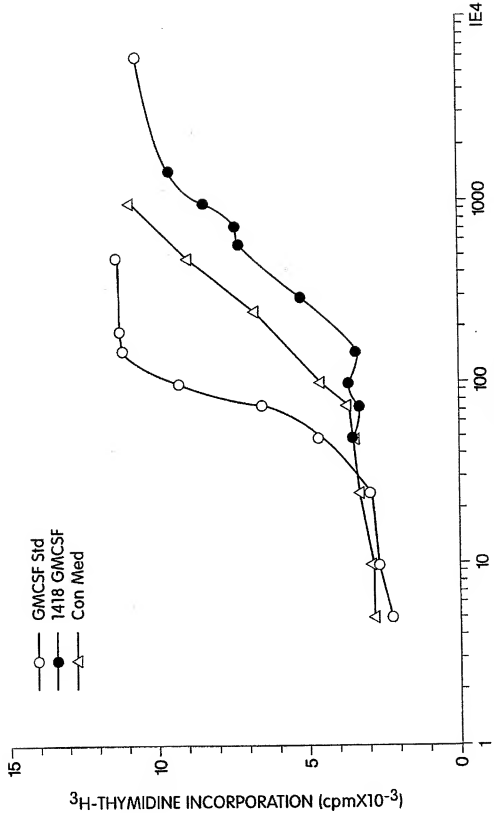


Fig. 8B


GMCSF Std
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Con Med
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10
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10
100
1000
IE4
GMCSF (pg/mL)

Fig. 9

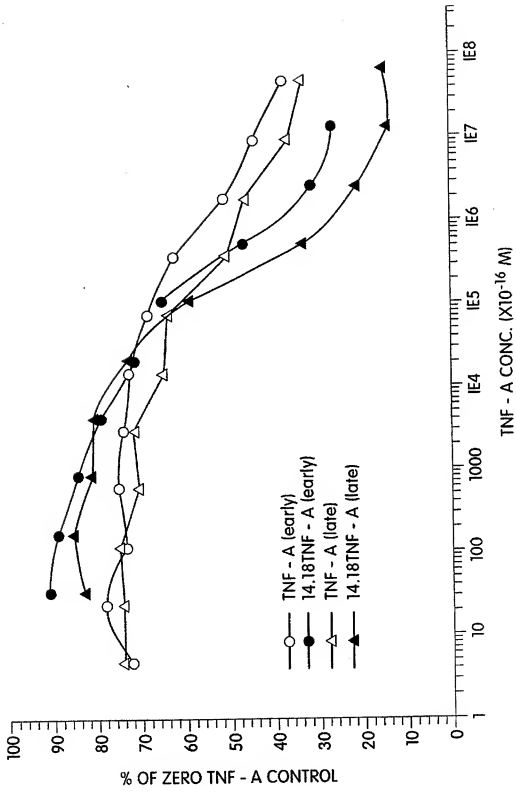
% OF ZERO TNF - A CONTROL
100
90
80
70
60
50
40
30
20
10
0
1
10
100
1000
IE4
IE5
IE6
IE7
IE8
TNF - A CONC. (X10-16 M)
TNF - A (early)
14.18TNF - A (early)
TNF - A (late)
14.18TNF - A (late)

Fig. 10

RECOMBINANT ANTIBODY CYTOKINE FUSION PROTEINS

This is a continuation of application Ser. No. 07/788,765 filed Nov. 7, 1991 (now abandoned), which is a continuation-in-part of application Ser. No. 07/612,099, filed Nov. 9, 1990 (now abandoned), the disclosures of which are incorporated herein by reference.

BACKGROUND OF THE INVENTION

The present invention relates generally to therapies involving the selective destruction of cells in vivo and to compositions of matter useful in the treatment of various cancers and vital infections. In particular, this invention relates to genetically engineered antibody fusion constructs capable of targeting an infected cell, and eliciting a localized inflammatory response such that the cell is killed or neutralized.

Tumor necrosis factor (TNFα) and lymphotoxin (LT or TNFβ) were first identified on the basis of their ability to directly kill certain tumors. However, many other biological activities are now attributed to these closely related cytokines. These include effects on a variety of cell types, such as the induction of histocompatibility antigens and adhesion receptors, as well as those resulting in inflammation, vascular permeability changes and mononuclear cell infiltration (Goeddel, D. V. et al. (1986) Symp. Quant. Biol. 51:597, Cold Spring Harbor; Beutler, B. and Cerami, A. (1988) Ann. Rev. Biochem. 57:505; Paul N. L. and Ruddle, N. H. (1988) Ann. Rev. Immunol. 6:407). The very short half-life of both TNFα and LT ensures that these inflammatory reactions do not occur systematically, but only at the sites of release from TNF-producing cells.

This ability to elicit a localized inflammatory response could be used in the treatment of solid tumors or other diseased tissue. For example, if it were possible to specifically deliver either TNFα or LT to a tumor site, a local inflammatory response could lead to an influx of effector cells such as natural killer cells, large granular lymphocytes, and eosinophils, i.e., the cells that are needed for antibody-dependent cellular cytotoxicity (ADCC) activity.

A way to deliver the lymphokine to a specific site in vivo is to conjugate it to an immunoglobulin specific for the site. However, the fusion of protein domains to the carboxy-termini of immunoglobulin chains or fragments can have unexpected consequences for the activities of both the protein to be fused and the immunoglobulin, particularly as far as antigen binding, assembly and effector functions are concerned. For example, the desired biological functions of the individual proteins may not be maintained in the final product.

Another potential problem with expressing proteins, such as the lymphokine LT, as a fusion protein to an immunoglobulin chain is that the native molecule exists in solution as a trimer and binds more efficiently to its receptor in this form. Thus, it seems unlikely that trimerization could still occur when LT is attached to an immunoglobulin heavy (H) chain via amino terminus and is assembled into an intact Ig molecule containing two paired H chain fusion polypeptides. Secondly, the ability of the fused LT to bind its receptor may be severely compromised if a free amino terminus is required for receptor binding activity. In fact, it has been postulated that the amino and carboxy-termini of TNFα, and, by analogy, LT, together form a structure that is required for receptor interaction.

It is an object of the invention to provide compositions of matter capable of selectively destroying cells in vivo, and

therapeutic methods for accomplishing this. It is also an object of the invention to provide compositions of matter and therapeutic methods for selectively delivering a cytokine to a target cell for the purpose of destroying the target cell either directly or by creating an environment lethal to the target cell.

SUMMARY OF THE INVENTION

This invention relates to immunoconjugates which include an immunoglobulin (Ig), typically a heavy chain, and a cytokine, and to the use of the immunoconjugates to treat disease. The immunoconjugates retain the antigen-binding activity of the Ig and the biological activity of the cytokine and can be used to specifically deliver the cytokine to the target cell.

The term "cytokine" is used herein to describe proteins, analogs thereof, and fragments thereof which are produced and excreted by a cell, and which elicit a specific response in a cell which has a receptor for that cytokine. Preferable cytokines include the interleukins such as interleukin-2 (IL-2), hematopoietic factors such as granulocyte-macrophage colony stimulating factor (GM-CSF), and tumor necrosis factor alpha (TNFα).

The term "lymphokine" as used herein describes proteins, analogs thereof, and fragments thereon produced by activated lymphocytes, and having the ability to elicit a specific response in a cell which has a receptor for that lymphokine, e.g., lymphotoxins. Lymphokines are a particular type of cytokine.

In preferred embodiments, the immunoconjugate comprises a chimeric Ig chain having a variable region specific for a target antigen and a constant region linked through a peptide bond at the carboxy terminus of the heavy chain to the cytokine.

Immunoconjugates of the invention may be considered chimeric by virtue of two aspects of their structure. First, the immunoconjugate is chimeric in that it includes an immunoglobulin chain (typically but not exclusively a heavy chain) of appropriate antigen binding specificity fused to a given cytokine. Second, an immunoconjugate of the invention may be chimeric in the sense that it includes a variable region and a constant region which may be the constant region normally associated with the variable region, or a different one and thus a V/C chimera; e.g., variable and constant regions from different naturally occurring antibody molecules or from different species. Also embraced within the term "immunoconjugate" are constructs having a binding domain comprising framework regions and variable regions (i.e., complementarity determining regions) from different species, such as are disclosed by Greg Winter et al., GB2, 188, 638. Preferably, the cytokine of the immunoconjugate can be a protein which naturally forms a dimeric or multimeric structure when unfused, such as LT or TNFα.

In a preferred embodiment, the chimeric Ig chain comprises a heavy (H) chain which includes the CH1, CH2 and CH3 domains. A proteolytic cleavage site may be located between the Ig heavy chain and the cytokine so that, when the conjugate reaches the target cell, the cytokine is cleaved from the heavy chain. A "proteolytic cleavage site" is an amino acid sequence recognizable by a protease with cleaves either within or proximal to the sequence. Preferably, the variable region is derived from a mouse (i.e. its DNA sequence or its amino acid sequence is based on a DNA or amino acid sequence of mouse origin) and the constant region (preferably including the framework region amino acids of the variable region) is derived from a human;

and the variable region of the heavy chain is derived from an Ig specific for a virus-infected cell, or for a tumor-associated or viral antigen. Preferably, the chimeric Ig chain can be assembled into the immunoconjugate by combining it with an appropriate counterpart (light or heavy) chain to form a monovalent antigen-binding region, which can then be associated to produce a divalent immunoconjugate specific for the target antigen.

The invention also features DNA constructs encoding the above-described immunoconjugates, and cell lines, e.g., myelomas, transfected with these constructs.

The invention also includes a method of selectively delivering a cytokine to a target cell, which method includes providing a cytokine immunoconjugate including a chimeric Ig chain including an Ig heavy chain having a variable region specific for the target cell and a constant region joined at its carboxy terminus by a peptide bond to a cytokine, and an Ig light chain combined with the chimeric Ig heavy chain, forming a functional antigen-binding site, and administering the immunoconjugate in an amount sufficient to reach the target cell to a subject harboring the target cell.

The invention thus provides an immunoconjugate in which the antigen binding specificity and activity of an antibody are combined in one molecule with the potent biological activity of a cytokine. An immunoconjugate of the invention can be used to deliver selectively a cytokine to a target cell in vivo so that the cytokine can exert a localized biological effect, such as a local inflammatory response, stimulation of T cell growth and activation, and ADCC activity. Such conjugates, depending on their specificity and biological activity can be used to treat diseases involving viral infections, or cancer, by targeted cell lysis, according to methods of the invention.

DESCRIPTION OF THE DRAWINGS

The foregoing and other objects of the present invention, and the various features thereof, may be more fully understood from the following description, when read together with the accompanying drawings, in which:

FIG. **1** is a schematic representation of one embodiment of the immunoconjugate of the present invention;

FIGS. **2A–D** are diagrams of the construction of fusion proteins between LT and the human Ig H chain; wherein FIG. **2A** is a map of a human Cγ1 gene fragment cloned in plasmid pBR322; FIG. **2B** shows the Cγ1 gene fused to LT at the end of the CH2 domain; FIG. **2C** shows the Cγ1 gene fused to LT at the end of the CH3 domain; FIG. **2D** shows the cDNA encoding LT cloned in expression vector pDEM including promoter (arrow), the natural leader peptide of LT (open box), the first residue of the mature protein (+1) and mouse κ L-chain poly A and 3'untranslated sequence. Open boxes represent protein coding regions of Cγ1 in A–C; black boxes represent synthetic linkers used to join the protein coding sequences; and striped boxes represent LT coding sequences;

FIG. **3** is a strippled representation of an SDS-polyacrylamide gel showing an analysis of fusion protein chain assembly, wherein chimeric ch14.18 antibody is shown in lanes **1** and **4**; CH2-LT is shown in lanes **2** and **5**; and CH3-LT is shown in lanes **3** and **6**. The position of stained marker proteins and their apparent molecular weights are indicated. The dried gel was exposed to film for either 4 hr (lanes **1** and **4**) or 18 hr. Cells were labeled with ^{35}S-methionine and secreted proteins were precipitated with an anti-human κ antiserum and protein A and analyzed on an SDS gel either reduced (lanes **1–3**) or unreduced (lanes **4–6**);

FIGS. **4A–B** are graphs showing the comparison of LT cytolytic activities for native LT (Δ--Δ), CH2-LT (o--o) or CH3-LT (o--o, filled in) immunoconjugates. A sensitive clone of the mouse fibroblast line **929** was used in the 1-day assay with mitomycin C. Relative cell survival was quantitated by staining with crystal violet and measuring the absorbance at 630 nm. FIG. **4A** shows culture supernatants from transfected cells assayed after first quantitating the conjugates by ELISA. FIG. **4B** shows purified proteins assayed following protein A Sepharose or immunoaffinity chromatography;

FIG. **5** is a graph of the effect of pH during purification on the cytostatic activity of CH3-LT. The activities of native LT (o--o), CH3-LT in culture supernatant (Δ--Δ), CH3-LT purified by protein A Sepharose chromatography (¤--¤) and CH3-LT purified at pH 6.5 (Δ--Δ) were compared in the cytostatic assay (in the absence of mitomycin C) using a mouse **929** subclone;

FIGS. **6A–B** are graphs of the cytolytic and cytostatic activities of LT and CH3-LT GD2-positive M21 human melanoma cells. M21 cells were seeded in 96-well plates in the [Bpresence (FIG. **6A**) or absence (FIG. **6B**) of mitomycin C and dilutions of LT (o--o) or CH3-LT (o--o, filled in) were added. Relative cell growth was measured by staining wells with crystal violet after 48 hr and measuring the absorbance at 630 nm;

FIG. **7** is a graph of the antigen binding activity of Ig/LT immunoconjugates. Relative binding was determined in a competitive antigen binding assay using ch14.18 antibody conjugated to HRP as tracer and either unlabeled ch14.18 (o--o), CH2-LT (o--o, filled in) or labeled ch14.18 (¤--¤) as competitor.

FIGS. **8A–B** are strippled representations of an SDS-polyacrylamide gel showing an analysis, under reducing (R) or nonreducing (NR) conditions, of the fusion protein **ch14.18**-CH3-GM-CSF (lane **1**) and the unfused protein ch14.18 (lane **2**), where M is molecular weight markers of indicated sizes.

FIG. **9** is a graph of GM-CSF activity of the Ig/GM-CSF immunoconjugate **ch14.18**-GM-CSF (o--o, filled in) compared to a GM-CSF standard (o--o) and conditioned medium (Δ--Δ).

FIG. **10** is a graph of TNF∝ activity of the Ig/TNF immunoconjugates **ch14.18**-TNF-∝ (early) (o--o, filled in), **ch14.18**-TNF-∝ (late) (Δ--Δ, filled in), compared to TNF-∝ (early) (o--o) and TNF-∝ (late) (Δ--Δ).

DETAILED DESCRIPTION OF THE INVENTION

The invention relates to immunoconjugates useful for killing a malignant or virus-infected target cell. The immunoconjugate includes a conjugate of an antibody portion having a specificity for a surface antigen on a virus-infected or malignant cell, and a cytokine.

FIG. **1** shows a schematic view of a representative immunoconjugate **10**. In this embodiment, cytokine molecules **2** and **4** are peptide bonded to the carboxy termini **6** and **8** of CH3 regions **10** and **12** of antibody heavy chains **14** and **16**. V_L regions **26** and **28** are shown paired with V_H regions **18** and **20** in a typical IgG configuration, thereby providing two antigen binding sites **30** and **32** at the amino ends of immunoconjugate **10** and two cytokine receptor-binding sites **40** and **42** at the carboxy ends of immunoconjugate **10**. Of course, in their broader aspects, the immunoconjugates need not be paired as illustrated.

The immunoconjugates of this invention can be produced by genetic engineering techniques; i.e., by forming a nucleic

acid construct encoding the chimeric immunoconjugate. Preferably, the gene construct encoding the immunoconjugate of the invention includes, in 5' to 3' orientation, a DNA segment which encodes a heavy chain variable region, a DNA segment encoding the heavy chain constant region, and DNA coding for the cytokine. The fused gene is assembled in or inserted into an expression vector for transfection of the appropriate recipient cells where it is expressed. The hybrid chain can be combined with a light (or heavy) chain counterpart to form monovalent and divalent immunoconjugates.

The cytokine can be any cytokine or analog or fragment thereof which has a therapeutically valuable biological function. Useful cytokines include the interleukins and hematopoietic factors such as interleukin-2 (IL-2) and granulocyte-macrophage colony stimulating factor (GMCSF). Lymphokines such as LT and TNFα, which require the formation of multimeric structures to function, can also be used. The gene encoding the lymphokine or cytokine can be cloned de novo, obtained from an available source, or synthesized by standard DNA synthesis from a known nucleotide sequence. For example, the DNA sequence of LT is known (see, e.g. Nedwin et al. (1985) Nucleic Acids Res. 13:6361), as are the sequences for interleukin-2 (see, e.g., Taniguchi et al. (1983) Nature 302:305–318), granulocyte-macrophage colony stimulating factor (see, e.g., Gasson et al. (1984) Science 266:1339–1342), and tumor necrosis factor alpha (see, e.g., Nedwin et al. 1. Ibid.)

The heavy chain constant region for the conjugates can be selected from any of the five isotypes: alpha, delta, epsilon, gamma or mu. Heavy chains or various subclasses (such as the IgG subclasses **1–4**) can be used. The light chains can have either a kappa or lambda constant chain. DNA sequences for these immunoglobulin regions are well known in the art. (See, e.g., Gillies et al. (1989) J. Immunol. Meth. 125:191).

In preferred embodiments, the variable region is derived from an antibody specific for the target antigen (an antigen associated with a diseased cell such as a cancer cell or virus-infected cell), and the constant region includes the CH1, CH2 and CH3 domains. The gene encoding the cytokine is joined, (e.g., by appropriate linkers, e.g., by DNA encoding $(Gly_4\text{-}Ser)_3$ in frame to the 3' end of the gene encoding the constant region (e.g., CH3 exon), either directly or through an intergenic region. In certain embodiments, the intergenic region can comprise a nucleotide sequence coding for a proteolytic cleavage site. This site, interposed between the immunoglobulin and the cytokine, can be designed to provide for proteolytic release of the cytokine at the target site. For example, it is well known that plasmin and trypsin cleave after lysine and arginine residues at sites that are accessible to the proteases. Many other site-specific endoproteases and the amino acid sequences they attack are well-known.

The nucleic acid construct can include the endogenous promoter and enhancer for the variable region-encoding gene to regulate expression of the chimeric immunoglobulin chain. For example, the variable region encoding genes can be obtained as DNA fragments comprising the leader peptide, the VJ gene (functionally rearranged variable (V) regions with joining (J) segment) for the light chain or VDJ gene for heavy chain, and the endogenous promoter and enhancer for these genes. Alternatively, the gene coding for the variable region can be obtained apart from endogenous regulatory elements and used in an expression vector which provides these elements.

Variable region genes can be obtained by standard DNA cloning procedures from cells that produce the desired antibody. Screening of the genomic library for a specific functionally rearranged variable region can be accomplished with the use of appropriate DNA probes such as DNA segments containing the J region DNA sequence and sequences downstream. Identification and confirmation of correct clones are then achieved by DNA sequencing of the cloned genes and comparison of the sequence to the corresponding sequence of the full length, properly spliced mRNA.

The target antigen can be a cell surface antigen of a tumor cell, a virus-infected cell or another diseased cell. Genes encoding appropriate variable regions can be obtained generally from Ig-producing lymphoid cells. For example, hybridoma cell lines producing Ig specific for tumor associated antigens or viral antigens can be produced by standard somatic cell hybridization techniques. (See, e.g., U.S. Pat. No. 4,96,265.) These Ig-producing cell lines provide the source of variable region genes in functionally rearranged form. The variable region genes will typically be of murine origin because this murine system lends itself to the production of a wide variety of Igs of desired specificity.

The DNA fragment containing the functionally rearranged variable region gene is linked to a DNA fragment containing the gene encoding the desired constant region (or a portion thereof). Ig constant regions (heavy and light chain) can be obtained from antibody-producing cells by standard gene cloning techniques. Genes for the two classes of human light chains and the five classes of human heavy chains have been cloned, and thus, constant regions of human origin are readily available from these clones.

The fused gene encoding the hybrid IgH chain is assembled or inserted into expression vectors for incorporation into a recipient cell. The introduction of gene construct into plasmid vectors can be accomplished by standard gene splicing procedures.

The chimeric IgH chain can be co-expressed in the same cell with a corresponding L chain so that a complete immunoglobulin can be expressed and assembled simultaneously. For this purpose, the heavy and light chain constructs can be placed in the same or separate vectors.

Recipient cell lines are generally lymphoid cells. The preferred recipient cell is a myeloma (or hybridoma). Myelomas can synthesize, assemble, and secrete immunoglobulins encoded by transfected genes and they can glycosylate protein. A particularly preferred recipient cell is the Sp2/0 myeloma which normally does not produce endogenous immunoglobulin. When transfected, the cell will produce only Ig encoded by the transfected gene constructs. Transfected myelomas can be grown in culture or in the peritoneum of mice where secreted immunoconjugate can be recovered from ascites fluid. Other lymphoid cells such as B lymphocytes can be used as recipient cells.

There are several methods for transfecting lymphoid cells with vectors containing the nucleic acid constructs encoding the chimeric Ig chain. A preferred way of introducing a vector into lymphoid cells is by spheroblast fusion. (see, Gillies et al. (1989) Biotechnol. 7:798–804). Alternative methods include electroporation or calcium phosphate precipitation.

Other useful methods of producing the immunoconjugates include the preparation of an RNA sequence encoding the construct and its translation in an appropriate in vivo or in vitro system.

The immunoconjugate of this invention can be used to deliver selectively a cytokine to a target cell in vivo so that the cytokine can exert a localized biological effect such as a

local inflammatory response, stimulation of T cell growth and activation, and ADCC activity. A therapeutically effective amount of the immunoconjugate is administered into the circulatory system of a subject harboring the target cell.

The invention is illustrated further by the following non-limiting Examples.

1. Plasmid Construction

Described below is the construction of PdHL2, a plasmid which contains the human Cγ1 heavy and kappa light chain gene sequences as well as insertion sites for V region cDNA cassettes (Gillies et al. (1989) J. Immunol. Meth. 125:191). This plasmid may be used as a starter plasmid for constructing any IgH chain cytokine fusion. For example, PdHL2 was used for the expression of Ig/LT fusion proteins. A LT cDNA was isolated from a human peripheral blood leukocyte library cloned in λgt10. The sequence was identical to that reported in the literature by Nedwin et al. (Nucleic Acids Res (1985) 13:6361). The cDNA was inserted into vector pDEM (Gillies et al., ibid) as an XhoI fragment after first removing most of the 3' untranslated region with Bal31 nuclease. The resulting plasmid, pDEM-LT (FIG. 2), expresses (in transfected cells) a fusion mRNA with a 5' untranslated sequence derived from the metallothionein (MT) promoter, the LT coding sequence and a 3' untranslated sequence and a poly A addition signal from the mouse Cκ gene. Fusion protein-encoding vectors were constructed by ligating HindIII to TaqI (CH2-LT) or HindIII to NsiI (CH3-LT) fragments of the human Cγ1 gene to HindIII and PvuII digested PDEM-LT using synthetic DNA linkers (FIG. 2). These linkers:

(5'-CGAAGAAAACCATCTCCAAA/CTCCCTGGTGTTGGCCTCAC
ACCTTCAG-3' (for CH2-LT); and

5-TGAGGCTCTCCACAACCACTACACGCAGAAGAGCCTCTCCCT
GTCCCCGGGTAAACTCCCTGGTGTTGGCCTCACACCTTCAG-3')

provide the protein coding sequence from the unique site (NsiI or TaqI) to the end of the heavy-chain domain (indicated by the slash), and join them to the amino terminus of the mature form of LT (up to the unique PvuII site). The linker for the CH3 fusion protein also includes a silent mutation that creates a SmaI site close to the end of the domain for future use in constructing fusion proteins. The DNA sequences at the junction of each construct were confirmed and each HindIII to EcoRI fragment was inserted into plasmid pdHL2-VCγ1κ(14.18). This plasmid contains the V cassettes for the ch14.18 anti-ganglioside GD2 antibody (Gillies et al., ibid.).

2. Cell Culture and Transfection

Sp2/0 Ag14 mouse hybridoma cells were maintained and transfected as described by Gillies et al. (BioTechnology (1989) 7:8799). Drug selection in methotrexate (MTX) was initiated 24 hours after transfection by adding an equal volume of medium containing MTX at 0.1 μM. Two additional feedings with selection medium were done at 3 day intervals. Transfectants secreting human Ig determinants were identified by ELISA (Gillies et al., 1989. ibid), grown in medium containing increasing concentrations of MTX, and subcloned by limiting dilution in medium containing MTX at 5 μM.

3. Purification and Characterization of Fusion Proteins

Proteins were biosynthetically labeled by incubating transfected cells (1×10^6/mL) for 16 hr in growth medium containing ^{35}S-methionine (50 μCi/mL-Amersham). Culture supernatants were then clarified by centrifugation in a microcentrifuge and the labeled proteins were immunoprecipitated with polyclonal anti-human κ chain antisera (Jackson Immunoresearch, Bar Harbor, Mc.) and protein A Sepharose (Repligen, Corp., Cambridge, Mass.). Protein samples were boiled for 5 min. in gel sample buffer in the presence or absence of 2-mercaptoethanol and analyzed on a 7% polyacrylamide gel. Proteins were detected by fluorography (diphenyloxazole in DMSO) and autoradiography.

Unlabeled proteins were purified from spent suspension culture medium by either immunoaffinity chromatography with a monoclonal anti-human κ antibody for the CH2-LT protein or by protein A Sepharose chromatography for the CH3-LT protein. All materials were concentrated by membrane dialysis into PBS. An alternative procedure for purification of the CH3-LT protein was developed to prevent the loss of LT activity during elution from the protein A column. Spent culture media was diluted with three volumes of 10 mM sodium phosphate buffer (pH 6.5) and loaded onto a Bakerbond AbX (J. T. Baker) column at room temperature. The column was washed with 10 mM sodium phosphate buffer until the absorbance returned to baseline and then with PBS, pH 6.5 (150 mM NaCl, 10 mM sodium phosphate, pH 6.5). The CH3-LT protein was eluted with 150 mM NaCl, 50 mM sodium phosphate, pH 6.5.

4. Activity Assay

The antigen binding activity of the Ig-LT proteins was measured as described in Gillies et al. (J. Immunol. Meth. (1989) 125:191), and LT activity was determined in the cytolytic or cytostatic assay (Kahn et al. (1982)) utilizing the 159124T2.5 subclone of the mouse L929 cell line (provided by Dr. H. Schreiber, University of Chicago). Cells were seeded into 96-well plates at 4×10^4 cells per well, with (cytolytic) or without (cytostatic) mitomycin C (2 μg/mL), and 10 μL of the test sample was added after 24 hr. Cells were stained either 24 or 48 hr later (see FIG. descriptions) with crystal violet and the amounts of dye retained in the wells were compared to those of untreated wells and those receiving the LT standard (R&D Systems). The same assay was also carried out with the GD2-bearing human melanoma line M21, originally provided by D. L. Morton, University of California, Los Angeles. The latter cell line was also used for measuring CDC and ADCC activity as described earlier (Gillies et al. (1990) Human Antibody Hybridomas 1:47)

5. Expression of Ig/LT Immunoconjugates

The Ig/LT immunoconjugates were made by directly fusing the cDNA sequence encoding the mature form of LT to the end of either the CH2 or CH3 exon of the human Cγ1 gene (FIG. 2) with the appropriate synthetic linkers. This gene fusion was then combined in a vector together with the V regions of murine antibody 14.18 and the human Cκ gene, and expressed in transfected Sp2/0 cells. These immunoconjugates were then expressed and tested for antigen binding activity and Ig chain assembly. The immunoconjugates retained antigen binding when measured in a competitive antigen binding ELISA (see below), and were assembled. Cells expressing these immunoconjugates were labeled with ^{35}S-methionine, and the secreted proteins were analyzed by SDS-PAGE in the presence or absence of reducing agent.

As seen in FIG. 3, the CH2-LT immunoconjugate was expressed as a mixture of whole (approximately 180 Kd) and half (90 Kd) molecules. The CH3-LT fusion protein, on the other hand, consisted entirely of fully assembled molecules. This result is not surprising since the CH3 domain is most responsible for Ig chain assembly. The reason why a portion of the CH2-LT did assemble, i.e. formed disulfide bonds in the hinge domain of the antibody, is likely due to the dimerization of the carboxy-terminal LT domains.

6. Biological Activity of Ig/LT Conjugates

The LT activities of the CH2-LT and CH3-LT conjugates were compared in the standard cytolytic assay (Kahn, A. et

al. (1982) "A standardized automated computer assisted micro-assay for lymphotoxin." In: *Human Lymphokines, Biological response modified*; (Kahn and Hill, eds.) Academic Press, New York. p. 23), using a mouse L929 subclone. This assay measures the ability of the immunoconjugate to bind to the TNF/LT receptor and trigger the active cell killing process in this cell line. When crude preparations (culture supernatants) were compared (FIG. 4A), CH3-LT was found to be much more active (nearly 100 fold by this assay) than CH2-LT and exhibited approximately the same specific activity per mole as the LT standard. This higher activity of CH3-LT is likely due to the increased proportion of fully assembled H-chain fusion proteins. Thus, the presence of the CH3 exon in the immunoconjugate may allow the H-chains to associate more efficiently, perhaps positioning the LT domains in a manner that allows for dimerization and, as a consequence, more LT receptor binding.

When purified preparations were compared, the difference in activities between CH2-LT and CH3-LT was still evident, but the activity of the conjugates, especially CH3-LT, was greatly reduced compared to the LT control (FIG. 4B). Since both proteins had been purified by using elution steps at acidic pH (i.e., less than pH4), the pH sensitivity of the culture supernatants was examined, and the LT activity was found to be very acid labile.

An alternative purification scheme was developed in which the pH was not reduced to below 6.5. The material from this preparation was compared to that purified by protein A, the original starting material, and the LT standard. The results of the LT cytostatic assay, in the absence of mitomycin C, shown in FIG. 5, demonstrate that full LT activity can be maintained during purification provided low pH is avoided. This assay was used to give a better dose response for the LT control and to demonstrate that the relationship between CH2-LT and CH3-LT is consistent for both assay systems. The same results were obtained in the cytolytic assay.

The results show that full activity (as measured by this assay) can be maintained when LT is fused to an Ig H chain. The fact that the LT amino terminus is covalently bound to the carboxy-terminus of the antibody apparently does not prevent LT receptor binding or the steps subsequent to binding that are required for activating the cell killing process.

7. Antigen Binding and Effector Functions of Ig/LT immunoconjugates

The antigen binding activity of the immunoconjugates was measured on antigen-coated plates in either a direct binding or competition assay format. In the direct binding assay antigen binding activity was found to be much higher than that of the control ch14.18 antibody. Since the source of the GD2 antigen was a crude membrane extract from neuroblastoma cells, it is possible that the TNF/LT receptor is present in the preparation and that binding of the conjugate through the LT domain is responsible for this increased activity. When antigen binding was measured in a competition assay, the conjugate was found to compete with the labeled ch14.18 antibody for antigen only slightly more efficiently than the unlabeled ch14.18 antibody (FIG. 7).

The results show that it is possible to combine the antigen binding activity of an anti-tumor cell antibody with the potent biological activity of a cytokine. The presence of the CH3 exon in the immunoconjugate results in complete H-chain assembly and, as a consequence, higher LT and effector activities. The assembly of H chains may likely result in LT dimerization.

In addition, a free amino terminus is not necessary for LT binding to its receptor since in the highly active CH3-LT immunoconjugate, the amino terminus of the LT domain is peptide bonded to the Ig H chain.

8. Construction and Expression of Ig/GM-CSF Immunoconjugates

Ig/GM-CSF conjugates were made by joining a nucleotide sequence encoding GM-CSF to a nucleotide sequence encoding an Ig heavy chain, such that the encoded protein includes a heavy chain fused via the carboxy terminus to GM-CSF. The construct was made as follows. The mature protein coding sequence of GM-CSF was linked to the end of the CH3 exon of the human $C\gamma_1$ gene using PdHDL2 and appropriate oligonucleotide linkers, as described above for the LT conjugate and according to procedures well-known in the art. Also as described above for LT conjugates, the Ig heavy chain GM-CSF fused gene was combined with the heavy chain V region gene of the 14.18 anti-GD2 heavy chain, and carried on the same vector as the human Cκ gene and the light chain V region gene of the 14.18 antibody. After transfection of the DNA into hybridoma cells and consequent expression of the H and L genes, a complete ch14.18 antibody with GM-CSF attached to the end of each H chain was produced. The fusion protein was purified from conditioned medium using adsorbtion to and elution from protein A Sepharose. The peak material was diafiltered using an Amicon stirred cell into PBS and concentrated to approximately 1 mg/mL.

The fusion protein was analyzed by electrophoresis on a 10% SDS-polyacrylamide gel (FIG. 8) under reducing (R) or non-reducing (NR) conditions and the proteins were visualized by staining with Coomassie Blue. Lane 1, ch14.18-CH3-GMCSF; Lane 2, ch14.18; M, molecular weight markers of the indicated sizes in kD. The relative molecular weight of the fused H chain of 75 kD in lane 1 (R) is consistent with a glycosylated GM-CSF (~25 kD) being fused to the H chain (50 kD). Note in the non-reduced lane 1 that the fusion protein is assembled into a single high molecular weight species of ~200 kD.

9. Biological Activity of Ig/GM-CSF Conjugates

The GM-CSF activity of the ch14.18-GM-CSF fusion protein was examined in a proliferation assay using the GM-CSF-dependent cell line AML-193 (human acute myelogenous leukemia) (obtained from Daniel Santoli, Wistar Institute, Philadelphia, Pa.). Cells are cultured for 2 days in serum-free medium containing insulin and transferrin (but no GM-CSF), at which time GM-CSF or fusion protein sample dilutions are added. After five more days, 5 μCi of ^{3}H-thymidine is added to each well and after 16 hr, the cells are harvested in 10% trichloroacetic acid. After 30 min. on ice the precipitated material is collected on GF/C filters, dried and counted by liquid scintillation.

In FIG. 9, the proliferation obtained with varying amounts of GM-CSF, conditioned medium containing the secreted fusion protein, or ch14.18-GM-CSF purified by protein A Sepharose are compared. The results show that significant GM-CSF activity is maintained once the molecule is fused to the H-chain but that the activity is either 20% (conditioned medium) or 10% that of (purified fusion protein) GM-CSF standard. Maximum incorporation was obtained with less than 10 ng/mL of the purified fusion protein (GM-CSF-equivalents or 50 ng of total protein). This slight loss of activity is not likely to affect the utility of this fusion protein, especially if large amounts of ch14.18-GM-CSF accumulate at the site of solid tumors expressing the GD2 antigen.

The in vivo half-life of the immunoconjugate was determined by injecting mice (20 pg injected in the tail vein) with ch14.18-GM-CSF. Samples of blood were collected at the

indicated times and the amount of fusion protein in the serum was determined by ELISA. The capture antibody was a polyclonal goat anti-human IgG (Fc-specific) and the detecting antibody was a horseradish peroxidase-conjugated goat anti-human K. As seen in Table 1, the half-life (calculated between the 24 hr and 4 day time points) was nearly 3 days. This compares to the published value of 85 min. in humans (Herrmann et al. (1989) J. Clin Oncol. 7:159–167). This increased half-life may compensate for the reduced activity of the fusion protein, especially since the local concentration of the immunoconjugate at the tumor site is likely to be increased by antibody targeting.

TABLE 1

Serum Concentration of ch14.18-CH3-GM-CS

Time after injection	Ab Concentration (ng/mL
4 hr	9210
16 hr	9660
24 hr	5950
4 days	2530

Mice were injected with 20 μg of the ch14.18-CH3-CSF fusion protein in the tail vein. Small samples (~50 μL) were taken from the tail vein and assayed for human antibody determinants.

10. Construction, Expression, and Activity of Ig/TNF Immunoconjugates

Ig/TNF immunoconjugates were made by fusing nucleotide sequences encoding TNFα and immunoglobulin heavy chain such that TNFα is fused to the carboxy terminus of the heavy chain. Briefly, the mature TNFα coding sequence was fused to the end of the human Cγ1 CH3 exon using oligonucleotides. The recombined fragment was joined downstream of the heavy chain V region encoding gene from the anti-GD2 mouse antibody 14.18; also contained in this vector was the human k gene, including both the V region gene encoding the light chain V region from the anti-GD2 mouse antibody 14.18 and the C region encoding gene. Hybridoma cells were transfected and selected as described above. Clones secreting human antibody determinants were expanded and used for the production and purification of the ch14.18-CH3-TNFα fusion protein by protein A Sepharose chromatography. The activity of the fusion protein was tested as described above for the CH3-LT fusion proteins.

As seen in FIG. 10, the amount of cytotoxicity obtained with the fusion protein met or exceeded that of native TNFα at either early (20 hr) or late (24 hr) points in the assay. This fusion protein appears to be fully functional with respect to TNFα activity, even though it was purified using protein A Sepharose. The CH3-LT construct was partially inactivated by the elution at acidic pH using the same protocol.

The results described above for the Ig/LT, Ig/GM-CSF, and Ig/TNFα immunoconjugates demonstrate that an antibody can be genetically fused to a cytokine without the loss of antigen binding activity and effector functions of the antibody, or the receptor binding and biological activity of a cytokine.

11. Dosage

Immunoconjugates of the invention may be administered at a therapeutically effective dosage within the range of lug-100 mg/kg body weight per day. The immunoconjugate may be administered in physiologic saline or any other biologically compatible buffered solution. This solution may be administered systemically (e.g., by injection intravenously or intramuscularly).

Other Embodiments

The invention may be embodied in other specific forms without departing from the spirit or essential characteristics thereof. The present embodiments are therefore considered to be in all respects as illustrative and not restrictive, the scope of the invention being indicated by the appended claims rather than by the foregoing description, and all changes which come within the meaning and range of equivalency of the claims are therefore intended to be embraced therein.

What is claimed is:

1. A recombinant immunoglobulin (Ig) chain comprising:

an Ig heavy chain and a cytokine which elicits a cytokine-specific biological response by a cell bearing a receptor for said cytokine, said Ig heavy chain comprising an N-terminal variable region specific for a cell bearing a cell surface antigen; and, CH1 and CH2 domains, said Ig heavy chain being joined at its carboxy-terminus by a peptide bond to the amino terminal amino acid of the cytokine.

2. The recombinant Ig chain of claim 1, wherein the Ig heavy chain further comprises the CH3 domain.

3. The recombinant Ig chain of claim 1, wherein a proteolytic cleavage site is located between the Ig heavy chain and the cytokine.

4. The recombinant Ig chain of claim 1, wherein the variable region is a mouse Ig variable region and the CH1 and CH2 domains are human Ig CH1 and CH2 domains.

5. The recombinant Ig chain of claim 1, wherein the variable region of the Ig heavy chain is specific for a cell surface antigen of a cancer cell or a virus-infected cell.

6. The recombinant Ig chain of claim 5, wherein said cell surface antigen is a tumor associated antigen or a viral antigen.

7. The recombinant Ig chain of claim 1, wherein the cytokine is tumor necrosis factor alpha.

8. The recombinant Ig chain of claim 1, wherein the cytokine is interleukin-2.

9. The recombinant Ig chain of claim 1, wherein the cytokine is a lymphokine.

10. The recombinant Ig chain of claim 9, wherein the lymphokine is a lymphotoxin.

11. The recombinant Ig chain of claim 9, wherein the lymphokine is granulocyte-macrophage colony stimulating factor.

12. The recombinant Ig chain of claim 9, wherein the lymphokine is a lymphokine which forms a dimeric or multimeric structure.

13. A cytokine immunoconjugate comprising:

(a) a recombinant immunoglobulin (Ig) heavy chain having an N-terminal variable region specific for a cell bearing a cell surface antigen of a cancer cell or a virus-infected cell, a constant region comprising CH1 and CH2 domains, and a cytokine joined to the carboxy-terminus of said constant region by a peptide bond, wherein said cytokine elicits a cytokine-specific biological response by a cell bearing a receptor for said cytokine; and

(b) an Ig light chain having a variable region specific for said cell surface antigen, said Ig heavy and light chains forming together a functional antigen-binding site, such that said immunoconjugate displays both antigen-binding specificity and cytokine biological activity.

14. The immunoconjugate of claim 13, wherein the recombinant Ig heavy chain has a constant region further comprising a CH3 domain.

15. The immunoconjugate of claim 13, wherein the cytokine is interleukin-2.

16. The immunoconjugate of claim 13, wherein the cytokine is tumor necrosis factor alpha.

17. The immunoconjugate of claim 13, wherein the cytokine is a lymphokine.

18. The immunoconjugate of claim 17, wherein the lymphokine is lymphotoxin.

19. The immunoconjugate of claim 17, wherein the lymphokine is granulocyte-macrophage stimulating factor.

20. The recombinant Ig heavy chain of claim 1 or 13 wherein said cytokine elicits a cytotoxic response by cells bearing a receptor for said cytokine.

21. The recombinant Ig heavy chain of claim 1 or 13 wherein said cytokine elicits a proliferative response by cells bearing a receptor for said cytokine.

* * * * *

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Unaltered immunoglobulin expression in hybridoma cells modified by targeting of the heavy chain locus with an integration vector

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SUMMARY

Chimeric antibodies against the murine T-cell antigen Thy-1.2 were generated in amounts sufficient for *in vivo* studies by substituting the constant gene segments via homologous recombination in the hybridoma cell. We show that an integration vector targets the heavy chain locus at high frequency even in a non-isogenic situation. Using this vector type, for the first time expression rates were obtained that were identical to the parental hybridoma. The use of the *gpt* selection marker seems to be crucial for efficient expression, and may overcome a recently claimed drawback of vector integration. A chimeric antibody produced by gene targeting was characterized *in vitro* and *in vivo*.

INTRODUCTION

To specifically design the effector functions of antibodies (Ab) and to reduce anti-antibody induction in clinical Ab therapy, chimerization and humanization strategies have been developed (review in ref. 1). Recombinant Ab are mostly expressed in non-producing myeloma cell lines transfected with immunoglobulin gene constructs. The production rates of these transfectants, however, being considerably lower than those of hybridoma cells, are a severe limitation for application in human therapy or in preclinical animal studies (ref. 2 and references within). It was assumed that the differential expression of endogenous and transferred immunoglobulin genes in B cells may be due to the lack of some *cis*-activating elements, e.g. the immunoglobulin 3′ enhancers,[3,4] in the commonly used expression vectors. However, the IgH 3′ enhancer had only a moderate effect on the expression level and could not restore the production rate of a hybridoma.[2] In contrast, very efficient expression of chimeric Ab can be obtained by introducing, by homologous recombination, the desired human constant (C) gene segments into the immunoglobulin loci of the hybridoma cell of interest thereby leaving all regulatory elements intact. Furthermore, this system is very time saving, as the variable (V) genes do not have to be isolated from the hybridoma and one recombination vector can be used for all specificities.

There are two types of recombination vectors:[5] replacement vectors, which mostly carry a two-sided homology flank neighbouring the heterologous region, and integration vectors, which usually contain one homology flank within which the construct is linearized, thus giving rise to a duplication of the target sequence. The exchange of C exons in the immunoglobulin loci has been achieved by using integration[6,7] as well as replacement constructs.[8–10] Integration vectors may have the advantages that they do not need a 3′ flank, which has to be matched to the isotype of the hybridoma, and that the requirement of just one cross-over renders them less sensitive to base pair mismatches.[11] Thus, targeting between sequences of different mouse strains should occur with higher fidelity and frequency.[11]

We have previously shown that chimeric Ab with human Fc regions and specificity for the murine pan T-cell antigen Thy-1.2 are capable of efficiently recruiting effector mechanisms in mice.[12] We then set out to study the immunosuppressive efficiency of these Ab with regard to skin graft survival in a preclinical mouse model. This required Ab amounts that could barely be provided in conventional transfection systems. So far, the expression rates of cell lines modified by gene targeting at the immunoglobulin loci are mostly inferior to those of the parental hybridomas, by at least a factor of two.[6,9,10] It was postulated that only replacement recombination provides the possibility to retain the original production rate.[13] Here we report on the generation of partially chimerized anti-Thy-1.2 Ab for *in vivo* studies; we show that an integration vector is actually highly efficient in a non-isogenic situation, and can produce cell lines that equal the expression level of the parental hybridoma.

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MATERIALS AND METHODS

Vector construction

The human IgG1 C region was ligated as a 2·9 kb *Eco*RI–*Pvu*II fragment[14] into *Eco*RI–*Bam*HI-digested pSV2gpt.[15] A 2·3 kb

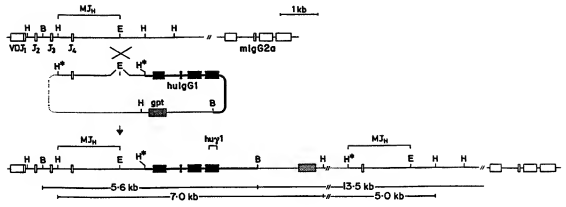

Figure 1. Genomic situation of the functionally rearranged IgH locus in the hybridoma MmT1, and predicted integration pattern induced by the vector pSVgpt–huγ1-A4. The hybridization probes are shown as brackets, the hybridizing fragments as bars. Open and closed boxes indicate mouse and human coding exons, respectively. Broken lines, *gpt* expression unit; stippled lines, pBR sequences. Restriction sites: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; the asterisk denotes an inactivated site.

*Hin*dIII fragment from the mouse μ intron was cloned into the *Eco*RI site of this construct, giving rise to pSVgpt–huγ1-A4. The construction of pSVgpt–huγ3-A4 was based on a 2·9 kb *Eco*RI–*Sph*I fragment carrying the human IgG3 C segments.[16] The vector design is shown in Fig. 1.

Cell-culture techniques

MmT1 is an AKR-derived hybridoma (γ2a/κ) with specificity for the murine Thy-1.2 antigen.[17] It was grown in RPMI-1640 medium (Gibco BRL, Eggenstein, Germany) supplemented with 10% fetal calf serum (FCS) and 2 mM glutamine. For transfection, 2×10^7 cells were suspended in 700 μl RPMI-1640 and mixed with 20 μg of the vector, which had been linearized with *Eco*RI. The cells were pulsed in a BioRad genepulser apparatus (220 V, 500 μF; München, Germany), kept at 0° for 10 min, and plated at a density of 10^4 cells/well in microtitre plates. After 48 hr, selection with 250 μg/ml xanthine, 15 μg/ml hypoxanthine and increasing amounts of mycophenolic acid (2 μg/ml final concentration) was initiated.

Immunological and in vivo *methods*

Supernatants of stably transfected clones were screened by enzyme-linked immunosorbent assay (ELISA) using goat anti-human IgGFc as capture Ab and peroxidase-labelled goat anti-human IgGFc as detection Ab (Dianova, Hamburg, Germany). The colour reaction was developed using o-phenylenediamine and measured in an ELISA reader at 405 nm. For the detection of mouse immunoglobulin, goat anti-mouse IgGFc was used. Quantitative ELISA were performed with purified human or mouse immunoglobulin as calibration proteins.[2] Alternately, Ab concentrations were determined by inhibiting binding to an anti-idiotypic Ab by biotinylated MmT1. This allowed quantification regardless of the isotypes involved. For Western blotting, Ab purified using protein G–Sepharose affinity chromatography was analysed using 8–15% SDS–polyacrylamide gradient gels. After electroblotting the proteins were detected by peroxidase-conjugated goat anti-human IgGFc.

Competitive binding studies were performed using a FACScan (Becton Dickinson, Heidelberg, Germany) calibrated with quantitative fluorescein microbead standards.

T-cell depletion *in vivo* was tested by injection of C57BL/6 mice with 400 μg of Ab intraperitoneally. Tail vein blood was taken on day 3. Blood cells were double-labelled with anti-CD3/anti-CD4, anti-CD3/anti-CD8 or anti-human IgGFc/anti-mouse μ and quantified in the FACS.

Hybridization procedures

Genomic DNA was prepared as described elsewhere.[18] Ten micrograms of DNA digested with various restriction enzymes was run on 0·7% agarose gels and blotted on Genescreen membranes (Du Pont, Boston, MA), which were hybridized with probes ^{32}P-labelled by random priming,[19] and washed under stringent conditions. The probe MJ_H is a 1·6 kb *Hin*dIII–*Eco*RI fragment including the mouse J_H4 segment; huγ1 is a 238 bp polymerase chain reaction (PCR) product derived from the human IgG1 C_H3 exon. (Fig. 1). Fluorescence in *in situ* hybridization was performed essentially as described elsewhere,[20] using a digoxigenin-labelled probe from the μ intron.

RESULTS

High-yield expression of recombinant anti-T-cell Ab following gene targeting with integration vectors

To study the T-cell depleting potential of human isotypes in a mouse model, the anti-Thy-1.2 Ab MmT1 was partially chimerized. The murine IgG2a C exons in the hybridoma MmT1 were exchanged with the human IgG1 or IgG3 region by homologous recombination. The insertion vectors pSVgpt–huγ1-A4 and pSVgpt–huγ3-A4 (Fig. 1) contain the human C exons, a murine 5′ homology flank and the *Ecogpt* marker. They were linearized within the homology region prior to transfection. Targeting events (Fig. 1) were enriched by selection with mycophenolic acid and detected by ELISA. Homologous recombination occurred at a frequency of 0·5%.

About 75% of the targeted clones continued to express the original mouse isotype. As suggested by Southern blot data (see below), this may be explained by the existence of at least two functional IgH copies. As homologous recombination is a rare

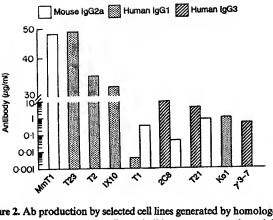

Figure 2. Ab production by selected cell lines generated by homologous recombination. Clones that did and did not coexpress the original mouse isotype are included. For comparison, the production rates of the parental hybridoma MmT1 and of the clones Ko1 and γ3–7 are also shown. The latter two transformants were obtained by conventional cotransfection of an H and L chain construct with MmT1 specificity into the non-producer cell line Sp2/0.[12]

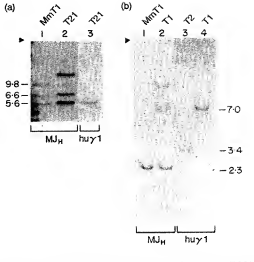

Figure 3. Southern blot analyses of representative clones. DNA from the indicated clones was digested with *Bam*HI (a) or *Hin*dIII (b) and hybridized with the indicated probes. The migration start points are marked by arrow heads. Sizes are given in kb. The 2·3 kb band in lane 1 of (b) represents all three alleles of MmT1 (Fig. 1) and did not disappear upon homologous recombination due to the presence of the aberrantly rearranged alleles, which remained unaltered.

event, clones only secreting the chimeric H chain are probably the result of a loss of the non-targeted copy. We could show, by sandwich ELISA, that the cells expressing two isotypes also secreted hybrid Ab that were composed of the H chains of two different species. Thus, formation of disulphide bridges between mouse IgG2a and human IgG1 and even IgG3 H chains is possible.

The amounts of Ab secreted were measured by quantitative ELISA or by protein determination after Ab purification. Even though there was considerable variation between different clones, in the high-level producers the IgG1 expression was in the range of the parental hybridoma (Fig. 2). Also, the transcript levels were comparable to those of MmT1 (data not shown). In the IgG1 low producers, also the coexpressed murine isotype was produced at low level. This may suggest a limitation of some *trans*-acting factors. Surprisingly, production of human IgG3 was consistently lower. However, this was also observed in conventional transfectants (γ3–7 in Fig. 2) and may be due to a less efficient transport of this isotype from the cell, as much protein could be detected in ELISA using lysates of IgG3-secreting cells.

Genomic situation in the targeted clones

MmT1 contains three H chain alleles (Fig. 3a, lane 1).[21] The 9·8 kb *Bam*HI band represents the functional allele, and the 6·6 kb fragment the aberrantly rearranged allele from the spleen cell; the 5·6 kb band originates from the fusion partner (our unpublished data). T21 was a clone having undergone an expected integration event. There was a novel 5·6 kb fragment that comigrated with that of the fusion partner and which was also detected by the huγ1 probe and a 13·5 kb band resulting from the duplication (Figs 1 and 3a, lanes 2 and 3). However, the functional allele had not disappeared but had just become weaker, which confirmed the existence of multiple gene copies. An *in situ* hybridization was performed to visualize the copies within the cell. We could, however, only detect three spots located on different chromosomes. This suggests that the copies of the functionally rearranged allele are separated by less than 100 kb.

We have demonstrated that integration vectors can initiate replacement-like events involving illegitimate recombinations.[7] We then examined 12 independent clones by Southern blotting and found that six showed this unexpected recombination pattern. For example, the clone T1 showed the predicted 7·0 kb *Hin*dIII fragment hybridizing with MJ_H, as well as with huγ1, but not the 5·0 kb band, which would be indicative for vector integration (Figs 1 and 3b, lanes 2 and 4).

It was postulated that gene replacement results in higher expression rates than vector integration, because it was found that replacement events separated the selection marker from the vector sequence and because a close proximity of the selection marker may exert an adverse effect on immunoglobulin transcription.[13] In some of our clones, for example T1 or T23, the presence of the 7·0 kb *Hin*dIII band indicated that the flank, the human C region and the *gpt* gene had not been separated from one another (Fig. 3b). The linkage was maintained irrespective of having undergone a replacement-like event. In accordance with the above-mentioned study,[13] T1 produced chimeric immunoglobulin in the range of just a few ng/ml, but other transformants such as T23 produced the human isotype at a rate identical to the parental hybridoma (up to 50 μg/ml). On the other hand, there were clones where the *gpt* gene had been integrated into an ectopic position, which was confirmed by various restriction digests and with several hybridization probes. Figure 3b (lane 3) shows the hybridization of *Hin*dIII-digested T2 DNA with huγ1, which detected a fragment of only 3·4 kb, although the corresponding vector sequence encompassing the C region and the *gpt* expression cassette spanned 4·7 kb. Despite the distant location of the selection marker, however, the production rate of T2 was reduced to about 70% compared with that of MmT1. Thus, the

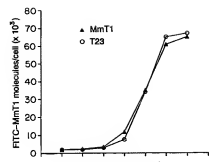

Figure 4. Inhibition of binding of FITC-labelled MmT1 to lymph node cells by serially diluted T23 or MmT1.

expression of chimeric immunoglobulin seems to be independent of the vicinity or absence of the *gpt* gene.

Characterization of the recombinant Ab *in vitro* and *in vivo*

For the *in vivo* studies the human IgG1 chimeric Ab T23 was selected, because IgG1 optimally activates murine effector mechanisms[12] and because this clone is a high-level producer and secretes no mouse Ab. The integrity of the Ab was confirmed by Western blotting (data not shown). The human C_H1 domain was shown to be able to pair with the original mouse κ chain. The antigen-binding avidity was tested in a competition assay. Binding of FITC-labelled MmT1 to Thy-1.2 on lymph node cells was inhibited by serially diluted unlabelled T23 or MmT1. T23 exhibited the same avidity as the original MmT1 (Fig. 4).

T-cell depletion induced by T23 in mice was very efficient. Only 0·9% T cells were found in the peripheral blood after Ab treatment (in comparison to 30% found in normal mice). This finding paves the way to assessing the immunosuppressive potential of T23 in the skin graft model.

DISCUSSION

To generate chimeric Ab for application in a mouse skin graft model, we introduced the desired alteration into the IgH locus of the hybridoma cell line by gene targeting. Our aim was to design a universal recombination system for the chimerization of IgH chains that should be applicable to any hybridoma regardless of the isotype and the mouse strain from which it is derived. Therefore, we used an integration construct, since this vector type needs no 3′ flank and yields higher recombination frequencies and fidelities when sequences non-isogenic to the vector flank are targeted.[11] Here we report for the first time the successful recombination between immunoglobulin sequences of different mouse strains (hybridoma from AKR, vector from BALB/c) and its application for the generation of a partially chimerized Ab for preclinical animal studies. The recombination efficiency (0·5%) was even higher than that reported for another comparable integration vector that was targeted to an isogenic IgH locus.[9] In some cases the integration vector promoted replacement-like events, but these events also mainly gave rise to functional chimeric Ab.

So far, the hybridoma cell lines generated by integration as well as replacement constructs have shown expression levels somewhat lower than those of the parental hybridomas.[6,9,10] Only in the case of gene replacement was the original production rate restored.[8,13] It was claimed that the selection marker which is posed into close proximity to the immunoglobulin expression unit upon vector integration, and which is transcribed from a promoter of its own, might interfere with immunoglobulin transcription.[13] This assumption does not hold true for our system, as in some clones (such as T23) the selection marker had been integrated into the IgH locus, but the expression rate of the parental hybridoma had remained unaltered. Conversely, other clones displayed a reduced production rate, although the selection marker had been separated from the immunoglobulin region. This discrepancy with the previous report[13] might be explained by the inclusion in our constructs of the *gpt* selection marker, instead of the *neo* gene which has been utilized in all other immunoglobulin recombination vectors described so far. Whether or not the original production rate can be achieved is not dependent on the nature of the recombination event, but rather on the use of the proper selection marker. We argue that the *gpt* gene does not impair the expression of chimeric immunoglobulin in *cis*, such as it was postulated for the *neo* gene.[10,13] Given the other advantages of integration vectors, the inclusion of the *gpt* marker renders this vector type even more suitable for routine application.

A problem generally encountered in the targeting of immunoglobulin loci is the frequent occurrence of clones that coexpress immunoglobulin molecules of two species. This finding can be explained by the existence of at least two copies of the functionally rearranged allele. In contrast to other investigators who claim that this is an exceptional feature,[10] we assume that the existence of multiple copies might be common to high-producing hybridomas, since it was observed in a variety of hybridomas[6,7,10] including a rat hybridoma.[7] Our *in situ* hybridization experiments suggest that the different copies are situated on the same chromosome.

Taken together, homologous recombination provides an efficient and convenient method for Ab chimerization. The expression levels obtained have been exceeded only by transfecting amplifiable vectors into non-producer cell lines.[22,23] However, gene targeting may be preferable because of the ease and rapidity of manipulation circumventing the need to isolate V genes, to perform selection schemes over periods of months, and to use toxic drugs.

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